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ABSTRACT OF DISSERTATION

Lisa Sue Middleton

The Graduate School
University of Kentucky
2005

NICOTINIC RECEPTOR MODULATION OF DOPAMINE TRANSPORTERS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By
Lisa Sue Middleton

Lexington, Kentucky

Director: Dr. Linda Dwoskin, Professor of Pharmaceutical Sciences
Lexington, Kentucky
2005

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ABSTRACT OF DISSERTATION

NICOTINIC RECEPTOR MODULATION OF DOPAMINE TRANSPORTERS

The current project examined the ability of nicotine to modulate dopamine transporter (DAT) function. Initial experiments determined the dose-response for nicotine to modulate dopamine (DA) clearance in rat striatum and medial prefrontal cortex (MPFC) using in vivo voltammetry and determined if this effect was mediated by nicotinic receptors (nAChRs). In both striatum and MPFC, nicotine increased DA clearance in a mecamylamine-sensitive manner, indicating nAChR-mediation. The effect of acute nornicotine on DAT function was also determined. In contrast to nicotine, nornicotine in a dose-related manner decreased striatal DA clearance in a mecamylamine-sensitive manner, indicating nAChR mediation. To determine if tolerance developed to the nicotine effect nicotine, separate groups of rats were injected once daily for 5 days with nicotine or saline. DA clearance in striatum and MPFC was determined 24 hrs after the last injection. Nicotine increased DA clearance only 10-15% in the group repeatedly administered nicotine, demonstrating that tolerance developed. To determine if nicotine altered striatal DAT efficiency, following nicotine injection,

DAT density and maximal velocity of [3 H]DA uptake was determined using [3 H]GBR12935 binding and saturation analysis of [3 H]DA uptake in rat striatum, respectively. Nicotine did not alter the B_{\max} or K_d of maximal binding of [3 H]GBR12935 binding. However, an increase in V_{\max} was observed at 10 and 40 min following nicotine injection, suggesting that nicotine increases DAT efficiency. To determine if systemic nicotine enhanced DAT function via an action at nAChRs on striatal DA terminals, [3 H]DA uptake was determined in striatum in vitro in the absence or presence of nicotine in the buffer. Nicotine did not alter the V_{\max} for [3 H]DA uptake in vitro, suggesting that the nicotine-induced increase in DAT function observed in vivo is mediated by nAChRs on DA cell bodies or another site which indirectly alters DAT function. To determine if the increase in DAT efficiency was due to increased surface expression of striatal DAT, biotinylation and Western blot analyses were performed. Nicotine did not alter striatal DAT, suggesting that the nicotine-induced increase in DA clearance in vivo and DAT efficiency in vitro is not the result of increased trafficking of this protein to the cell surface.

KEYWORDS: Nicotine, Nornicotine, Nicotinic Receptors, Dopamine Transporters, Striatum, Medial Prefrontal Cortex

Lisa Sue Middleton

December 22, 2005_____

NICOTINIC RECEPTOR MODULATION OF DOPAMINE TRANSPORTERS

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Name

Date

DISSERTATION

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Acknowledgments

I would like to take this opportunity to thank my mentor Dr. Linda Dwoskin for the guidance and direction she has given me. I am grateful to her for being my mentor and friend. My committee has challenged me to be successful and I feel prepared to move forward in this field. Thank you Dr. Subbu Apparsundaram, Dr. Wayne Cass, Dr. Lisa Cassis, Dr. Peter Crooks and Dr. Jim Pauly. I would also like to thank Dr. Bruce O'Hara for agreeing to serve as the outside examiner for my dissertation defense.

During my time at the University of Kentucky I met my husband, JC. Thank you for all of your love and emotional support – especially during those moments of stress and deadlines.

To my family and friends – thank you for many years of support. To my parents for never giving up on me, my sister and my friends for providing the laughs and distractions when needed.

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Chapter One

Introduction and Background

I. Drug Abuse and Reward

The brain is a complex organ made up of various structures that interact with each other through neuronal networks. Drugs of abuse have many different effects on neuronal circuitry and can modulate how information is carried throughout the brain. Psychostimulants, such as nicotine, cocaine and amphetamine, modulate two primary circuitry pathways, the mesolimbic and nigrostriatal pathways. Figure 1 represents a sagittal section through a rat brain. The mesolimbic system has cell bodies in the ventral tegmental area (VTA) and sends projections to the nucleus accumbens and the medial prefrontal cortex (MPFC). The nigrostriatal pathway has cell bodies in the substantia nigra (SN) and sends projections to the striatum. The mesolimbic and nigrostriatal pathways are dopaminergic pathways, i.e., dopamine (DA) is the neurotransmitter contained in the neurons in this pathway.

Previous research on the neurobiology of reward and drug addiction has focused on the mesocorticolimbic and nigrostriatal DA systems, emphasizing the role of the nucleus accumbens, MPFC and striatum. The accumbens shell, which is innervated by dopaminergic projections from the VTA, and its associated neurocircuitry are believed to encode primary appetitive stimuli associated with unconditioned drug reward (Wise and Bozarth, 1987; Robbins and Everitt, 1996; Bardo, 1998; Koob, 1999; Di Chiara, 2000; Kelley and Berridge, 2002), including

reward produced by nicotine (Fibiger and Phillips, 1987; Corrigall et al., 1992, 1994; Balfour and Benwell, 1993; Mansvelder and McGehee, 2002; Mathieu-Kia et al., 2002). More recently, focus has been placed on the involvement of the striatum and MPFC in reward and reinforcement. The MPFC, which includes the anterior cingulate cortex and is innervated by dopaminergic projections from the VTA, is believed to encode secondary conditioned stimuli associated with environmental cues paired with drug, leading to reward expectancy, which is recognized as important to the process of addiction and relapse to drug use (Berridge and Robinson, 1998; Shima and Tanji, 1998; Kelley, 1999; Di Chiara, 2000; Kelley and Berridge, 2002; Peoples, 2002; Cohen et al., 2004; Di Chiara et al., 2004; Rose and Behm, 2004; Brody et al., 2004). Integration of the motivational information from the MPFC occurs at least in part in striatal cholinergic neurons, which are innervated by dopaminergic projections from the substantia nigra, leading to the initiation and execution of movement in reward expectancy and detection of reward (Martin-Soelch et al., 2001).

Ia. Animal Models of Reward

According to the DSM-IV, drug abuse is defined as an individual who uses a drug despite the knowledge that that drug may be harmful to them. Abuse is different from drug dependence/addiction in that an addicted individual has an obsessive preoccupation with a drug, i.e., the drug is the most important relationship the individual has. Animal models have been developed to examine the abusive or rewarding properties of drugs. Conditioned place preference is a

paradigm in which an environmental stimuli is paired with a reward such as food or drug. This results in a preference of the animal for the environment that was paired with the reward, even in the absence of the reward. Therefore, one can determine the rewarding properties of a drug by evaluating the animal's preference for the environment associated with reward. Drug self-administration is the "gold-standard" for measuring the rewarding properties of a drug. In this paradigm, animals are trained to press a lever to receive a drug reward. Fixed-ratio (FR) self-administration is a reinforcement schedule that will vary the number of lever presses required to receive a reward. For example, an FR1 means that one lever press will result in one infusion of drug whereas an FR5 schedule requires 5 lever presses to receive one infusion of drug. The number of lever presses can be increased within a session (progressive ratio schedule) which will require the animal to work harder to receive the reward. The point at which an animal will no longer increase the number of bar presses to receive drug is known as the break point, indicating that the level that the animal will work for the drug, indicating the amount of reward associated with it.

II. Diseases and Disorders

Tobacco smoking and depressive disorders are highly comorbid (Glassman et al., 1993; Covey et al., 1997; Covey, 1999; Pomerleau et al., 2000; Cardenas et al., 2002). Nicotine has been reported to have antidepressant properties in depressed individuals (Glassman, 1993; Salin-Pascual and Drucker-Colin, 1998; Balfour and Ridley, 2000; Picciotto et al., 2002) and in

animal models of depression (Tizabi et al., 1999; Vazquez-Palacios et al., 2004). Such evidence has led to the self-medication hypothesis of nicotine dependence, such that individuals may use tobacco, at least in part, to ameliorate depression (Markou et al., 1998; Balfour and Ridley, 2000; Hughes et al., 2003; Popik et al., 2003). The self-medication hypothesis is further strengthened by the observation that antidepressants, bupropion and perhaps others, serve as an efficacious smoking cessation pharmacotherapies (Hurt et al., 1997; Jorenby et al., 1999; Cryan et al., 2003a,b; Ferry and Johnston, 2003; Hausteiner, 2003; Richmond and Zwar, 2003; Hall et al., 2004), although the mechanism of action in this regard is not clear. Clinical depression is generally accepted as associated with dysfunction of monoaminergic neurotransmitter systems. Most effective antidepressants inhibit monoamine transporter function, increasing the extracellular concentrations of DA, norepinephrine and/or serotonin. Many antidepressants, including bupropion (Wellbutrin®), inhibit monoamine transporters, but also act as nicotinic receptor antagonists (Richelson and Pfenning, 1984; Nomikos et al., 1989; Ascher et al., 1995; Fryer and Lukas, 1999; Slemmer et al., 2000; Li et al., 2002; Miller et al., 2002a,b; Gumilar et al., 2003; Damaj et al., 2004). Smokers treated with bupropion while trying to quit smoking have been shown to have a decrease in symptoms of depression (Lerman et al., 2004).

The use of nicotine replacement as a cessation therapy is based on activation of nicotinic receptors resulting in DA release, and potentially nicotine's

ability to modulate DAT function. Treatment of smokers with a transdermal nicotine patch has been shown to increase positive affect, whereas nicotine administration by a nasal spray did not have any impact on affect (Strasser et al., 2005), which demonstrates the importance of not only type of treatment but route of administration. Furthermore, the presence of a DAT polymorphism (SLC6A3), in cigarette smokers, has been associated with an increase in stress-induced craving associated with smoking (Erblich et al., 2004). That is exposure to a stressor results in an increased craving for a cigarette, and the stress-induced craving is higher in smokers with the SLC6A3 DAT polymorphism. One possibility is that both transporters and nicotinic receptors contribute to antidepressant alleviation of depression as well as to smoking cessation. Understanding functional interactions between DAT and nicotinic receptors may provide a mechanistic basis and more thorough comprehension of the relationship between depression and nicotine dependence.

A number of neurological diseases result in an alteration in the number of nicotinic receptors in striatum and SN, e.g., Parkinson's disease and schizophrenia. In Parkinson's disease, there is a selective loss of nigrostriatal DA neurons with a concomitant loss of presynaptic nicotinic receptors on DA nerve terminals in striatum (Wonnacott et al., 1997). In post mortem striatal tissue from Parkinson's patients, there is a decrease in the number of [³H]nicotine binding sites (Court et al., 2000; Guan et al., 2002). Furthermore, a decrease in the number of $\alpha 3/\alpha 6$ nicotinic receptors in monkey brain has been

shown after nigrostriatal damage (McCallum et al., 2005), as well as a loss of nicotine binding in the substantia nigra (Perry et al., 1995). Following chronic tobacco use in humans, there is an increase in the number of nicotine binding sites in the brain (Perry et al., 1999). Furthermore, people who smoke tobacco are reported to have a lower incidence of Parkinson's disease (Morens et al., 1995; Baron, 1986; Gorell et al., 1999; Quik and Kulak, 2002). Both tobacco smoke and nicotine have been shown to be protective against MPTP neurotoxicity, which is an animal model for Parkinson's (Parain et al., 2003). Therefore, therapies that increase the number of nicotine binding sites or nicotinic receptors may be beneficial in the treatment of Parkinson's disease.

Schizophrenic patients have a higher incidence of tobacco smoking compared to the general population (Hughes et al., 1986; Lohr and Flynn, 1992; Dalack et al., 1998; Esterberg and Compton, 2005). A lack of sensory gating is observed in schizophrenics and this may involve desensitization of nicotinic receptors (Griffith et al., 1998). In striatum, nicotine binding sites are increased in schizophrenic patients (Court et al., 2000). Interestingly, hypoactivity is observed in the dopaminergic neurons in prefrontal cortex in schizophrenic patients (Davis et al., 1991), suggesting that different brain regions and potentially different nicotinic receptor subtypes in these brain regions may be involved in schizophrenia. In schizophrenic smokers, nicotine administration improved delayed recognition memory, visuospatial working memory and continuous performance test scores (Myers et al., 2004; Sacco et al., 2005).

More recently, schizophrenic patients with a 113 base pair allele of the $\alpha 7$ nicotinic receptor gene were found to be more likely to smoke and smoke heavily (De Luca et al., 2004). In addition, a polymorphism in the $\alpha 7$ nicotinic receptor gene (CHRNA7) was found to be linked to the auditory gating deficit common in the schizophrenic patient population (Freedman et al., 1997). Consistent with its proposed involvement in sensory gating, selective $\alpha 7$ nicotinic receptor agonists and partial agonists such as tropisetron have been shown to restore auditory gating deficits in rodent models of schizophrenia (Stevens et al., 1998; O'Neill et al., 2003; Hajós et al., 2005; Koike et al., 2005; Siegel et al., 2005). Not only nicotinic receptors, but also inhibition of monoamine transporters by bupropion has been shown to improve the auditory gating deficits in a mouse model of schizophrenia (Siegel et al., 2005).

III. Dopamine

Dopamine (Figure 2) is a neurotransmitter in the central nervous system. Figure 3 shows a schematic representation of a DA nerve terminal. The first step in the synthesis of DA is the conversion of tyrosine to 3, 4-dihydroxyphenylalanine (DOPA) in the DA nerve terminal by the enzyme tyrosine hydroxylase. Tyrosine is an amino acid that is transported across the blood brain barrier and then into DA neurons by amino acid transporters. DOPA is then converted to DA by DOPA decarboxylase in the nerve terminal. Tyrosine hydroxylase is the rate-limiting enzyme of DA synthesis. Once DA is synthesized, it can be stored in synaptic vesicles in preparation for release from

the terminal into the synapse. Following stimulation and depolarization of the nerve terminal, synaptic vesicles fuse to the synaptic membrane and release DA into the synaptic cleft by a process called exocytosis. After release, DA can be metabolized by catechol-O-methyl transferase or monoamine oxidase in the synapse, bind to presynaptic D2 DA autoreceptors located presynaptically, activate postsynaptic D1 and D2 DA receptors, or be transported back into the terminal by the DA transporter (DAT; Cooper et al., 2003). D1 and D2 DA receptors are the most abundant DA receptors in striatum. The D1 DA receptor has one known subtype, D5, which acts similarly to D1 in that it stimulates adenylate cyclase activity, but D5 has a 10-fold higher affinity for DA than does D1. D1 is located throughout the brain including striatum and cortex. However, D1 is more abundant in striatum than cortex. D5 is also only located in hippocampus, thalamus and hypothalamus. The D2 DA receptor family also has two receptor subtypes, D3 and D4. Similar to D2 receptors, D3 and D4 both inhibit adenylate cyclase activity. However, D3 has a 100-fold higher affinity for the DA receptor agonist quinpirol, and D4 has an order of magnitude higher affinity for the atypical antipsychotics such as clozapine. D2 is located in both striatum and cortex and is also more abundant in striatum than cortex. Both D3 and D4 receptors have very little expression in the striatum or MPFC. Once back inside the terminal, DA can be re-stored in vesicles or metabolized by monoamine oxidase located in the cytoplasm to provide the metabolite dihydroxyphenylacetic acid (DOPAC).

In smokers, nicotine dose-dependently increases neuronal activity in cingulate cortex, MPFC, striatum and accumbens assessed using functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) (Stein et al., 1998; Barrett et al., 2004). Recently, in an fMRI study examining decision-making and monetary reward, activity in the striatum was correlated with short-term reward (Haruno et al., 2004). These data suggest that the striatum is heavily involved in reward-based behavioral learning. Changes in striatal neural activity and blood flow have been observed following rewarding experiences in studies using humans (Delgado et al., 2000; Knutson et al., 2000; Elliot et al., 2003; O'Doherty et al., 2004; Tanaka et al., 2004; Tricomi et al., 2004), monkeys (Kawagoe et al., 2003; Watanabe et al., 2003; Yamada et al., 2004), and rats (Schmitzer-Torbert and Redish, 2004). The changes in neural activity may be related to the magnitude of reward that is received (Cromwell and Schultz, 2003). Recently, injection of a kappa-opioid antagonist into mouse striatum was shown to result in a decrease in cocaine-induced conditioned place preference, also indicating the involvement of the striatum in the rewarding properties of cocaine (Zhang et al., 2004).

IV. The Dopamine Transporter (DAT)

Brain monoamine transporters are expressed almost exclusively in neurons that contain their cognate transmitter. Immunolocalization studies reveal the presence of DAT, the norepinephrine transporter and the serotonin transporter on their respective cell bodies, axons and terminals (Nirenberg et al.,

1996; Hersch et al. 1997; Hoffman et al., 1998; Sesack et al. 1998; Schroeter et al., 2000; Mengual and Pickel, 2004). As shown in Figure 4, DAT is a twelve transmembrane protein with both the carboxy and amino termini located intracellularly. Translocation of DA by DAT across the membrane is coupled with the cotransport of two Na^+ ions and one Cl^- ion down their electrochemical gradients, generating a small inward current (Kanner and Schuldiner, 1987; Rudnick and Clark, 1993). Moreover, the DA neuron membrane potential influences DAT function, such that hyperpolarization increases the velocity of DA transport by DAT and depolarization decreases DA transport velocity (Sonders et al., 1997). DA is also removed from the extracellular space by metabolism and diffusion, the latter factor playing a larger role in brain regions with sparse dopaminergic innervation and lower DAT density, such as the MPFC (Sesack et al., 1998). These transporter proteins are located presynaptically and may be in close apposition to nicotinic receptors, suggesting the potential for their functional interaction. Psychostimulants and antidepressants interact with these transporters (Barker and Blakely, 1996). For example, amphetamine reverses monoamines transporters evoking monoamine release, whereas cocaine and antidepressants inhibit transporter function. Since nicotine is a psychostimulant with antidepressant properties, it is reasonable to hypothesize that nicotine may interact with or modulate transporter function.

DAT is a major pharmacological target for psychostimulant action.

Psychostimulants inhibit DAT function and increase synaptic DA concentrations

(Horn 1990). Cocaine and amphetamine alter the expression of cell surface DAT protein (Saunders et al. 2000; Daws et al. 2002; Little et al. 2002). In cell expression systems, cocaine increases cell surface expression of DAT, whereas amphetamine decreases DAT localization on the cell surface. The ability of nicotine to modulate the cell-surface expression of DAT has not been examined. As many psychostimulants alter DAT localization as their mechanism of action, studies examining the ability of nicotine to modulate DAT localization are warranted. DAT is regulated by activation of protein kinase C, which decreases the maximal velocity (V_{\max}) of DA uptake and decreases DAT cell surface expression in cell expression systems and in brain (Melikian et al. 1994; Copeland et al. 1996; Melikian et al. 1996; Huff et al. 1997; Vaughan et al. 1997; Zhang et al. 1997; Pristupa et al. 1998; Daniels and Amara 1999; Melikian and Buckley 1999; Chi and Reith 2003). Little is known about the effect of nicotine on transporter function and trafficking, as well as the underlying cellular mechanisms regulating these processes.

IVa. DAT Regulation through Trafficking

Studies suggest that monoamine transporters are regulated by several mechanisms, including constitutive internalization and recycling (i.e., trafficking), involving transporter phosphorylation and protein-protein interactions (Zahniser and Doolen, 2001; Kahlig and Galli, 2003; Loder and Melikian, 2003; Torres et al., 2003; Melikian, 2004). Psychostimulants and second messengers alter phosphorylation, function and trafficking of these transporters (Gnegy et al.,

2004; Kahlig et al., 2004). For example, DAT surface expression in cell lines is acutely sensitive to amphetamine and cocaine, which decrease and increase DAT surface levels, respectively (Saunders et al., 2000; Daws et al., 2002; Little et al., 2002; Zahniser and Sorkin 2004). Protein kinase C activation in native preparations and cell systems decreases surface distribution of DAT (Vaughan et al., 1997; Zhang et al., 1997; Zhu et al., 1997; Daniels and Amara, 1999; Melikian and Buckley, 1999; Loder and Melikian, 2003), whereas protein kinase A and calcium-calmodulin kinase II activation results in upregulation of DAT at the cell surface (Page et al., 2004). Transporters undergo internalization and recycling, which may involve dynamin-clathrin mediated pathways and multiple protein-protein interactions, such as interaction with syntaxin-1A, PP2A, PICK1, ERK and synuclein (Zahniser and Doolen, 2001; Torres et al., 2003; Lee et al. 2004; Melikian, 2004; Valjent et al., 2005). Furthermore, psychostimulants can modulate DAT function by phosphorylation of DAT. For example, amphetamine activates protein kinase C and mitogen activated protein kinase which enhances amphetamine stimulated DA release through DAT (Park et al., 2003; Johnson et al., 2005). DAT can also be modulated by interneuronal signaling such as nitric oxide (Kiss et al., 2004), which can inhibit DAT function. Nitric oxide is able to diffuse between different neurons and thus provides a mechanism for other neurotransmitter systems to modulate DAT.

V. Nicotine

Nicotine (Figure 2) is the most common drug of abuse and tobacco dependence the leading preventable cause of death in the USA (Surgeon General's Report, 1988; Jaffe, 1990; USDHS, 2001). Despite the deleterious effects of tobacco, ~23% of the US population continues to use tobacco (Trosclair et al., 2002). About 70% of smokers want to quit, ~40% make an attempt to quit, however, only ~5% are successful at cessation for a period of 3-12 months (Trosclair et al., 2002). Nicotine, the most abundant alkaloid in tobacco (Bush et al., 1993), has intrinsic rewarding properties, which are believed to be responsible for tobacco dependence (Koob, 1992; Corrigall et al., 1992; Stolerman and Jarvis, 1995; Di Chiara, 2000; Balfour, 2002; Mathieu-Kia et al., 2002; Garrett et al., 2003). Volunteers who habitually smoke and are tobacco deprived will self-administer nicotine (Henningfield and Goldberg, 1983; Harvey et al., 2004; Johnson et al., 2004). Indicative of its reinforcing properties, nicotine is self-administered in animal models using both fixed-ratio schedules (Goldberg et al., 1981; Henningfield and Goldberg, 1983; Cox et al., 1984; Corrigall and Coen, 1989; Corrigall et al., 1992, 1994; Sannerud et al., 1994; Donny et al., 1998, 2003; Rose and Corrigall, 1997; Shoaib et al., 1997; Valentine et al., 1997; Rasmussen and Swedberg, 1998; Caggiula et al., 2001, 2002; Suto et al., 2001; LeSage et al., 2002, 2003; Fu et al., 2003; Paterson et al., 2004) and progressive-ratio schedules (Donny et al., 1999; Bruijnzeel and Markou, 2003). While the maintenance of smoking is believed to be due to the reinforcing

properties of nicotine, it is also possible that people continue to smoke to avoid the withdrawal symptoms instead of for reinforcement.

Some of the rewarding properties of nicotine are considered beneficial such as mood elevation, arousal and learning and memory enhancement (Clarke, 1987; Pomerleau and Pomerleau, 1992). The mechanisms underlying the reinforcing properties of nicotine are not well understood, but are important for understanding the initiation and maintenance of tobacco smoking behavior. Although the mechanisms underlying nicotine's reinforcing properties are not well understood, the mesocorticolimbic and nigrostriatal DA systems are believed to be involved. For example, activation of the immediate early gene, c-fos, in the anterior cingulate cortex, nucleus accumbens and striatum has been demonstrated in nicotine self-administering rats (Pagliusi et al., 1996; Merlo-Pich et al., 1997). Additionally, cortical c-fos has been shown to be activated following exposure to environmental cues associated with repeated nicotine administration (Schroeder et al., 2001). The latter results from animal studies are consistent with findings showing that in tobacco smokers, nicotine dose-dependently increases neuronal activity in the cingulate cortex, frontal lobe and nucleus accumbens, as determined using functional MRI (Stein et al., 1998).

Elucidating the mechanism of nicotine action and determining the effects of chronic nicotine administration are of importance in understanding the initiation and maintenance of tobacco smoking behavior and in overcoming the difficulties

habitual smokers experience in extinguishing this behavior. As previously discussed, evidence for the reinforcing properties of nicotine includes studies reporting nicotine self-administration by many species on various reinforcement schedules (Goldberg et al., 1981; Henningfield and Goldberg, 1983; Cox et al., 1984; Corrigall et al., 1992, 1994; Sannerud et al., 1994; Donny et al., 1996) and nicotine-induced conditioned place preference in rats (Fudala et al., 1985; Shoaib et al., 1994; Risinger and Oakes, 1995). Studies clearly indicate that under an FR5 schedule as well as when nicotine is given to an animal contingently, nicotine is avidly self-administered by rats (Corrigall and Coen, 1989; Corrigall et al., 1992, 1994; Donny et al., 1996). Also, nicotine self-administration, both fixed- and progressive-ratio, is decreased in animals and humans by mecamylamine and other nicotinic receptor antagonists indicating nicotinic receptor involvement (Meltzer and Rosecrans, 1981; Stolerman et al., 1973, 1984; Corrigall et al., 1994a; Rose et al., 1994; Mansbach et al., 2000; Shoaib et al., 1997, 2002, 2003; Markou and Paterson, 2001; Glick et al., 2002; Mansvelder and McGehee, 2002; Bruijnzeel and Markou, 2003; Rauhut et al., 2002, 2003).

Concomitant with the intrinsic reinforcing effects of nicotine, there is strong evidence indicating that nicotine stimulates locomotor behavior. In drug-naïve rats, acute nicotine administration produces an initial depressant effect on activity followed by hyperactivity lasting 1 hour or longer (Clarke and Kumar, 1983; Clarke, 1990). With chronic nicotine administration, tolerance develops to the

transient hypoactive phase (Stolerman et al., 1973, 1974; Collins et al., 1988, 1990; Martin et al., 1990), whereas sensitization develops with respect to the hyperactive phase (Clarke and Kumar, 1983; Ksir et al., 1985, 1987; Clarke et al., 1988; Fung and Lau, 1988; Schoaib and Stolerman, 1992; Benwell and Balfour, 1992; Ksir, 1994; Stolerman et al., 1995). Mecamylamine blocks both the hypoactive and hyperactive phases following acute nicotine injection and also blocks the development of behavioral sensitization occurring following chronic nicotine administration (Clarke and Kumar, 1983). Drugs of abuse, including nicotine, amphetamine and cocaine, have been suggested to produce their locomotor stimulant and reinforcing effects by activating the nigrostriatal and mesolimbic DA systems, respectively (Fibiger and Phillips, 1987; Corrigall et al., 1992, 1994; Balfour and Benwell, 1993). This pathway regulates cognitive and emotional behaviors (Simon et al., 1980) and is particularly sensitive to the acute effects of psychostimulants (Roberts and Koob, 1983; Wise and Bozarth, 1987; Self and Nestler, 1995). Furthermore, chronic nicotine exposure increases the maximal binding (B_{max}) of nicotinic receptors (Collins et al., 1990; Bhat et al., 1991, 1994; Marks et al., 1992; Sanderson et al., 1993), demonstrating nicotine-induced upregulation of nicotinic receptors.

VI. Nicotinic Receptors

Extracellular DA concentrations are increased following nicotine stimulation of nicotinic acetylcholine receptors in the cell body and terminal regions of the mesocorticolimbic and nigrostriatal systems. Nicotinic receptors

are a family of ligand-gated transmembrane ion channels that are activated physiologically by acetylcholine (Figure 2) and pharmacologically by nicotine. Both acetylcholine and nicotine activate all known nicotinic receptor subtypes, whereas mecamylamine noncompetitively inhibits all known subtypes (Varanda et al., 1985; Loiacono et al., 1993; Peng et al., 1994). Mecamylamine (Figure 2) is a nonselective, noncompetitive nicotinic receptor antagonist that blocks the opening of nicotinic receptors by binding in the pore of the receptor (Varanda et al., 1985; Loiacono et al., 1993; Peng et al., 1994). Nonselective antagonists are not specific for a certain subtype of receptor, in the case of mecamylamine, it inhibits all nicotinic receptor subtypes. Noncompetitive antagonists are antagonists that do not compete for the agonist binding site on the receptor, but act at an allosteric site on the protein removed from the agonist recognition site. Therefore, noncompetitive inhibition cannot be overcome by increasing the agonist concentration. While much is known about the function of nicotinic receptors, the specific subunit composition of native nicotinic receptors in brain has not been elucidated. Therefore, native nicotinic receptors in brain are only putatively designated.

Once these receptors are activated, they undergo a transition from the closed, resting conformation to an open state, conducting cations (Na^+ , K^+ , Ca^{++}) through the channel down their electrochemical gradients, causing rapid depolarization of the target cell (McGehee and Role, 1995; Role and Berg, 1996; Alkondon et al., 1997; Dani et al., 2001). Heteropentameric nicotinic receptors

require two molecules of acetylcholine or nicotine to bind in order for the ion channel to open, whereas, homopentamers can bind up to five molecules of acetylcholine or nicotine.

As shown in Figure 5, nicotinic receptors are pentameric structures composed of nine different alpha ($\alpha 2 - \alpha 10$) and three different beta ($\beta 2 - \beta 4$) subunits, with a general stoichiometry of 2α and 3β (Luetje et al., 1990; Anand et al., 1991; Cooper et al., 1991; Deneris et al., 1991; Luetje and Patrick, 1991; Role, 1992; Sargent, 1993; Elgoyhen et al., 1994; McGehee and Role, 1995; Elgoyhen et al., 2001). $\alpha 7$ nicotinic receptors are homopentamers thus containing 5 different $\alpha 7$ subunits. Amino acid sequencing determined that nicotinic receptor subunits have four membrane spanning regions (M1-M4). The M2 region from each of the five subunits is believed to form the wall of the pore in the assembled functional receptor (Galzi and Changeux, 1995). Mutation of the M2 region has been shown to alter the Ca^{++} permeability of $\alpha 7$ nicotinic receptors (Bertrand et al., 1992). The alpha subunits are identified by a pair of adjacent cysteine residues in the amino terminal domain, and are thought to be the agonist binding subunits (Jensen et al., 2005). The beta subunit appears to be a codeterminant of the functional properties of the receptor (Cachelin and Rust, 1995; Jensen et al., 2005). While much is known about the function of nicotinic receptors, the specific subunit composition of native nicotinic receptors in brain

has not been elucidated. Therefore, native nicotinic receptors in brain are only putatively designated (indicated by an asterisk).

Hybridization cloning has revealed a surprising degree of subtype diversity among neuronal nicotinic receptors (Luetje et al., 1990; Luetje and Patrick, 1991; Deneris et al., 1991; Role, 1992; Sargent, 1993; Elgoyhen et al., 1994; McGehee and Role, 1995). The $\alpha 2$, $\alpha 3$ and $\alpha 4$ subunits form functional receptors with either $\beta 2$ or $\beta 4$ when coexpressed in *Xenopus* oocytes. In contrast, $\alpha 7$, $\alpha 8$ and $\alpha 9$ form functional homo-oligomers in oocytes (Couturier et al., 1990; Séqu  la et al., 1993; Elgoyhen et al., 1994; Gerzanich et al., 1994; Peng et al., 1994; Briggs et al., 1995, 1998; Chavez-Noriega et al., 1997; Rothlin et al., 1999). $\alpha 7$ nicotinic receptors are highly permeable to Ca^{++} and have fast desensitization kinetics in the presence of agonist. The $\alpha 5$, $\alpha 6$ and $\beta 3$ subunits do not form functional receptors on their own or in combination with another single subunit in oocytes. However immunoprecipitation studies indicate that in *vivo*, $\alpha 5$ complexes with $\alpha 3$ and $\beta 4$, and more recently, that $\alpha 3$ combines with $\alpha 4\beta 2$ and $\alpha 4\beta 4$, suggesting that more than two different subunits may assemble together forming a receptor, which greatly increases the potential diversity of native nicotinic receptors (Conroy et al., 1992; Keyser et al., 1993; Vernallis et al., 1993; Ramirez-Lattore et al., 1996; Forsayeth et al., 1995; Wang et al., 1996; Campos-Caro et al., 1997; Forsayeth and Kobrin, 1997). The functional significance of subtype diversity is still unknown; however, both native and cloned subtypes are pharmacologically

distinct in their response to both nicotinic agonists and antagonists (Luetje and Patrick, 1995; Luetje et al., 1990; Role, 1992; Luetje, 1993; Amar et al., 1993; Sargent, 1993; Cachelin and Rust, 1995; Decker, 1995).

Heteromeric $\alpha 4\beta 2^*$ nicotinic receptors are most abundant in brain, and second most abundant are homomeric $\alpha 7^*$ nicotinic receptors (Whiting and Lindstrom, 1986; Wada et al., 1989; Morris et al., 1990; Schoepfer et al., 1990; Anand et al., 1991; Flores et al., 1992; Lukas et al., 1999). The subunits display different, but overlapping, patterns of expression in brain (e.g., $\beta 2$ mRNA expression is prominent in the cerebellum, which exhibits no $\alpha 4$ hybridization; Wada et al., 1989). mRNA for nine subunits ($\alpha 2$ – $\alpha 7$ and $\beta 2$ – $\beta 4$) of nicotinic receptors have been identified in SN and VTA dopaminergic neurons (Klink et al., 2001, Zoli et al., 2002; Azam et al., 2002; Wooltorton et al., 2003), indicating that potentially a large number of heteromeric nicotinic receptor subtypes of pentameric structure may be expressed by these neurons.

Nicotinic receptors are localized on presynaptic terminals and cell bodies of VTA and SN DA neurons (Schwartz et al., 1984; Clarke and Pert, 1985; McGehee and Role, 1995; Wonnacott, 1997; Quik et al., 2005); and evidence indicates that nicotinic receptor subtypes at cell body and terminal locations differ pharmacologically (Reuben et al., 2000; Champiaux et al., 2003). In striatal synaptosomes, nicotine has a lower affinity for nicotinic receptors than acetylcholine, and acetylcholine has equal affinity to epibatidine (a nicotinic

receptor agonist; Reuben et al., 2000). However, in a dendrosomal preparation, nicotine has equal affinity to epibatidine (Reuben et al., 2000), suggesting that the nicotinic receptors in the terminal regions differ from those in the cell body regions. Furthermore, $\alpha 6\beta 2^*$ receptors have been shown to be located on DA terminals, whereas non $\alpha 6(\alpha 4\beta 2)^*$ receptors represent the majority of functional receptors on DA cell bodies (Champtiaux et al., 2003). The selective $\alpha 6$ nicotinic receptor antagonist, α -conotoxin MII, has been shown to decrease nicotine-evoked DA release, suggesting the involvement of the $\alpha 6$ subunit in nicotine-evoked DA release (Salminen et al., 2004). Both the α -conotoxin MII sensitive and resistant nicotinic receptor subtypes required the $\beta 2$ subunit, however only the α -conotoxin MII sensitive subtype required the $\beta 3$ subunit; whereas the α -conotoxin MII resistant subtypes required $\alpha 4$. These data suggest that the primary subtypes involved in DA release are the $\alpha 6\beta 3\beta 2^*$ and $\alpha 4\beta 2^*$ subtypes (Salminen et al., 2004). Importantly, α -conotoxin MII inhibits nicotine-evoked [3 H]DA overflow by only 50%, implicating involvement of at least two different nicotinic receptor subtypes in this response (Zoli et al., 2002; Salminen et al., 2004; Azam and McIntosh 2005). It is likely that the difference in pharmacology between the nicotinic receptors in the cell body regions and the nerve terminal regions indicates that these receptors play different physiological roles.

VII. Nicotine Modulation of DA System Function

The pharmacological effects of nicotine are complex. Nicotine can stimulate release of many different hormones including prolactin, vasopressin

and adrenocorticotropin (Cam et al., 1979; Conte-Devolx et al., 1981; Andersson et al., 1983; Mendelson et al., 2003; Sun et al., 2005). Furthermore, nicotine increases blood pressure and heart rate (Benowitz et al., 1982; Benowitz et al., 1988; Kadoya et al., 1994; Fattinger et al., 1997), and increases cerebral blood flow (Kodaira et al., 1992; Stein et al., 1998; Ernst et al., 2001; Rose et al., 2003; Domino et al., 2004). To add to the complexity of nicotine dependence, genetics as well as socioeconomic status play important roles in the development of dependence to cigarette smoking (Boomsma et al., 1994; Breslau et al., 2001; Berrettini and Lerman, 2005). Nicotine not only releases DA as described above, but also releases other neurotransmitters such as norepinephrine and serotonin (Yoshida et al., 1980; Toth et al., 1992; Sharp and Matta, 1993; Yu and Wecker, 1994; Rao et al., 2003; Shearman et al., 2005). While nicotine has many different effects, the current project will focus on the effects of nicotine on the DA system.

Nicotine-evoked DA release is observed at nicotine concentrations (0.1-0.8 μ M) within the range found in smokers' blood (Russell et al., 1980; Kogan et al., 1981). Nicotine increases DA release in striatum, nucleus accumbens and MPFC in vitro (Giorguieff-Chesselet et al., 1979; Arqueros et al., 1978; Westfall, 1974; Westfall et al., 1983; Chesselet, 1984; Rowell et al., 1987; Rapier et al., 1988, 1990; Fung, 1989; Westfall et al., 1989; Izenwasser et al., 1991; Grady et al., 1992, 1994; Harsing et al., 1992; Rowell and Hillebrand, 1994; Schulz et al., 1993; El-Bizri and Clarke, 1994; Rowell, 1995; Sacaan et al., 1995; Teng et al.,

1997; Puttfarcken et al., 2000; Wonnacott et al., 2000; Zhou et al., 2001; Grady et al., 2002; Mogg et al., 2002; Wilkins et al., 2002; Grinevich et al., 2003; Crooks et al., 2004; Dwoskin et al., 2004; Rice and Cragg, 2004; Zhang and Sulser, 2004). Nicotine also increases DA release in striatum, accumbens and MPFC in vivo (Imperato et al., 1986; Damsma et al., 1989; Brazell et al., 1990; Toth et al., 1992; Benwell et al., 1993, 1995; Bassareo et al., 1996; Marshall et al., 1997; Maisonneuve and Glick, 1999; Di Chiara, 2000; Fu et al., 2000; Sziraki et al., 2002; Tizabi et al., 2002; Bednar et al., 2004; Brody et al., 2004b; Rahman et al., 2004). Systemic nicotine administration also markedly increases the firing rate of mesolimbic DA neurons recorded extracellularly (Grenhoff et al., 1986).

Importantly, nicotinic receptors localized to cell bodies mediate nicotine-induced DA release locally (somatodendritic), as well as at terminals (Clarke et al., 1988; Corrigall et al., 1994; Nisell et al., 1994a,b 1995; Fu et al., 2000; Sziraki et al., 2002; Tizabi et al., 2002; Champtiaux et al., 2003; Rahman et al., 2003; Laviolette and van der Kooy, 2003). Nicotine-evoked DA release is inhibited by mecamylamine and/or di-hydro beta-erythroidine (a competitive nicotinic receptor antagonist; DH β E), and is Ca⁺⁺ dependent (Westfall et al., 1987; Rapier et al., 1990; Grady et al., 1992; El-Bizri and Clarke, 1994; Nisell et al., 1994; Crooks et al., 1995; Saccaan et al., 1995; Rowell, 1995; Teng et al., 1997). Pre-exposure of striatal slices or synaptosomes from mice or rats with nicotine results in functional desensitization, such that subsequent nicotine exposure produces a

diminished [^3H]DA release (Rapier et al., 1988; Grady et al., 1994; Rowell and Hillebrand, 1994; Rowell, 1995). The desensitization induced by nicotine is reversible, and is observed even with pre-exposure concentrations which produce no detectable stimulation of [^3H]DA release.

Controversy exists regarding the exact subunit composition of nicotinic receptor subtypes mediating nicotine evoked DA release, and great effort is focused on elucidating specific nicotinic receptor subtypes involved. Using electrophysiology, a hypertonic sucrose solution allows for measurement of the size of the readily releasable pool of vesicles from synaptosomes (Turner, 2004). Repeated application of nicotine to the synaptosomes increased DA release by increasing the size of the readily releasable pool of vesicles. This effect is inhibited by α -bungarotoxin, suggesting a role for the $\alpha 7^*$ nicotinic receptor subtype in nicotine-evoked DA release (Turner, 2004). Subtype assignment of native nicotinic receptors mediating nicotine evoked DA release has been based largely on inhibition of agonist-induced response by subtype selective antagonists defined by inhibitory activity in cell systems expressing nicotinic receptor subtypes of known composition (Grady et al., 2002; Zoli et al., 2002; Everhart et al., 2004; Azam and McIntosh, 2005), by results from studies using nicotinic receptor subunit knockout mice (Picciotto et al., 1998; Champtiaux et al., 2002, 2003; Whiteaker et al., 2002; Marubio et al., 2003), and by in situ hybridization and single cell polymerase chain reaction of mRNA in DA cell

bodies (Deneris et al., 1989; Wada et al., 1989; Le Novere et al., 1996; Charpentier et al., 1998; Klink et al., 2001; Azam et al., 2002).

Initially, specific subunit combinations of nicotinic receptors mediating nicotine-evoked DA release (Imperato et al., 1986; Vezina et al., 1992; Nisell et al., 1996, 1997; Teng et al., 1997; Zhou et al., 2001) were thought to be the $\alpha 3\beta 2^*$ (Schulz and Zigmond, 1989; Luetje and Patrick, 1991; Grady et al., 1992; Cartier et al., 1996; Kaiser and Wonnacott, 2000) and $\alpha 4\beta 2^*$ (Grady et al., 1992; Rapier et al., 1990; Kulak et al., 1997; Kaiser and Wonnacott, 2000; Sharples et al., 2000) nicotinic receptor subtypes. More recently, the selective $\alpha 6$ nicotinic receptor antagonist, α -conotoxin MII, has been shown to decrease DA release, suggesting the involvement of the $\alpha 6$ subunit in nicotine-evoked DA release (Salminen et al., 2004). As previously discussed, DA neurons express $\alpha 3$ - $\alpha 7$ and $\beta 2$ - $\beta 4$ mRNAs (Deneris et al., 1989; Wada et al., 1989; Le Novere et al., 1996; Charpentier et al., 1998; Klink et al., 2001; Azam et al., 2002) resulting in a number of nicotinic receptor subtypes and subunit combinations that may be involved in nicotine-evoked DA release. For example, α -conotoxin MII is a nicotinic receptor antagonist, which inhibits electrophysiological responses in *Xenopus* oocytes expressing $\alpha 3/\alpha 6$ and $\alpha 6/\alpha 4$ nicotinic receptors containing either $\beta 2$ or $\beta 4$ subunits (Luetje et al., 1990; Cartier et al., 1996; Kuryatov et al., 2000; Luetje, 2004). Importantly, α -conotoxin MII inhibits nicotine-evoked [3 H]DA overflow by only 50%, implicating involvement of at least two different nicotinic receptor subtypes (Zoli et al., 2002; Salminen et al., 2004; Azam and McIntosh

2005). Immunoprecipitation of nicotinic receptors from DA terminals as well as electrophysiological studies in DA neurons from various nicotinic receptor knockout mice suggest the involvement of $\alpha 4\beta 2^*$, $\alpha 6\beta 2^*$, $\alpha 6\alpha 4\beta 2^*$ nicotinic receptors in DA release (Champtiaux et al., 2003). Furthermore, striatal lesion with 6-OHDA determined that possibly $\alpha 3$ -, $\alpha 4$ - or $\alpha 5$ -containing nicotinic receptors were present on DA terminals and could be involved in nicotinic receptor modulation of DA release (Zoli et al., 2002). α -Conotoxin MII binds with high affinity to immunopurified $\alpha 6\beta 2$ nicotinic receptors (Zoli et al., 2002; McIntosh et al., 2004), which is eliminated in $\alpha 6$ -knockout mice, but not in $\alpha 3$ - or $\alpha 4$ -knockout mice (Champtiaux et al., 2002; Whiteaker et al., 2002; Marubio et al., 2003); suggesting that α -conotoxin MII is a selective antagonist for $\alpha 6$ containing receptors. Nicotine does not evoke DA release in microdialysis experiments using $\alpha 4$ -knockout mice (Marubio et al., 2003), indicating that $\alpha 4$ nicotinic receptors are critical for DA release, at least in ventral striatum. However, the latter study also reported that basal DA release in $\alpha 4$ -knockout mice was significantly greater than in the wild-type, suggesting that $\alpha 4$ -knockout resulted in a compensatory response (altered tonic activity). Evidence is also emerging suggesting that different DA neurons can be categorized based upon the expression of particular nicotinic receptor subtypes with varying compositions of nicotinic receptor subunits (Azam et al., 2002). Chronic nicotine administration to $\beta 2$ nicotinic receptor knockout mice did not increase locomotor activity, which is suggested to be a DA dependent behavior. Thus the $\beta 2$ subunit plays an important role in DA-dependent behavior (King et al., 2004). Thus, we are only

beginning to obtain a comprehensive understanding of the heterogeneous population of nicotinic receptors that mediate nicotine-evoked DA release and DA-dependent behaviors.

VIII. Nicotine Modulation of DAT

Extracellular DA concentrations are the net result of exocytotic DA release from presynaptic terminals and reuptake of DA into presynaptic terminals (clearance) via DAT. Nicotine-induced stimulation of nicotinic receptors results in depolarization of the plasma membrane (Calabresi et al., 1989), which would be expected to decrease DAT function and decrease DA clearance, thereby increasing extracellular DA concentrations. Many psychostimulants act at DAT as part of their mechanism of action. Thus, studies determining the effect of nicotine on DAT function are important to pursue.

Recently, $\alpha 4$ nicotinic receptor subunit knockout mice have been shown to have a decreased DAT function compared to wild type mice (Parish et al., 2005). These data suggest that nicotinic receptors may be involved in modulating basal DAT function. Furthermore, nicotine (0.4 mg/kg) has been reported to increase DA clearance (enhance DAT function) in the nucleus accumbens in anesthetized rats (Hart and Ksir, 1996). The latter results also contrast findings *in vitro* in which nicotine and another nicotinic agonist, 1,1-dimethyl-4-phenyl-piperazinium, have been reported to decrease [3 H]DA uptake into chopped striatum and PC12 cells, respectively (Izenwasser et al., 1991; Huang et al., 1999). However, these

in vitro findings have not been replicated (Kramer et al., 1989; Carr et al., 1989; Rowell and Hill 1993; Zhu et al., 2003).

The observation that nicotine enhances DAT function in nucleus accumbens *in vivo* (Hart and Ksir, 1996) is unexpected considering the electrogenic nature of the transporter. As described previously, translocation of DA by DAT across the membrane is coupled to the cotransport of two Na⁺ ions and one Cl⁻ ion down their electrochemical gradients, generating a small inward current (Kanner and Schuldiner, 1987; Rudnick and Clark, 1993; Hitri et al., 1994). Moreover, the DA neuron membrane potential influences DAT function, such that hyperpolarization increases the velocity of DA transport by DAT and depolarization decreases DA transport velocity (Sonders et al., 1997). As such, nicotine-induced stimulation of nicotinic receptors, which results in depolarization of the plasma membrane (Calabresi et al., 1989), would be expected to decrease DAT function and decrease DA clearance, thereby increasing extracellular DA concentrations. Recently DAT has been shown to act as a channel, not just as a transporter (Carvelli et al., 2004). This would suggest that DAT has another role and can itself modulate membrane potential.

With respect to nicotinic receptor modulation of DAT function, nicotine has also been reported to enhance amphetamine-evoked [³H]DA release from MPFC slices incubated in assay buffer in the absence of calcium (Drew et al., 2000). These assay conditions precluded nicotine-evoked exocytotic DA release,

suggesting the involvement of DAT in the nicotine-induced augmentation of the response to amphetamine. Surprisingly, nicotine did not enhance amphetamine-evoked [^3H]DA release in striatum or nucleus accumbens (Drew et al., 2000), indicating that this effect was specific to MPFC. The observed nicotine enhancement of the effect of amphetamine in MPFC was inhibited by nicotinic receptor antagonists, mecamylamine and DH β E, but not by α -bungarotoxin, indicating that specific, non $\alpha 7$ containing, nicotinic receptor subtypes are involved (Drew et al., 2000). The latter findings suggest that nicotinic receptors may modulate DAT function under physiological conditions, i.e., in the presence of extracellular calcium, at least in MPFC. Thus, determining the ability of nicotine to modulate DAT function in striatum and MPFC, areas involved in reward, is important for understanding the rewarding properties associated with tobacco smoking and nicotine self-administration.

IX. Specific Aims and Hypotheses:

The work presented in this dissertation was based on the formation of four specific aims: 1) Determine the dose-response relationship for systemic nicotine to modulate DAT function in striatum and MPFC via a nicotinic receptor-mediated mechanism; 2) Determine the effect of repeated peripheral nicotine administration on DAT function in striatum and MPFC to assess differential brain-region specific nicotine-induced regulation of DAT using *in vivo* voltammetry; 3) Determine if an increase in DAT efficiency is responsible for the nicotine-induced increase in DAT function; and 4) Determine if increased trafficking of DAT from

internal sites to the presynaptic terminal membrane accounts for the increase in DA clearance in response to nicotine administration.

Specific Aim 1

Hypothesis #1

Nicotine will increase DA clearance in the striatum and MPFC in a dose-dependent manner, and this increase in clearance will be mediated by nicotinic receptors.

Hypothesis #2

Nornicotine, similar to nicotine, will increase DA clearance in the striatum via a nicotinic receptor-mediated mechanism.

Specific Aim 2

Hypothesis #3

Repeated nicotine administration will further enhance the nicotine-induced increase in DA clearance in striatum and MPFC.

Specific Aim 3

Hypothesis #4

Systemic pretreatment with nicotine will increase DAT function in striatal synaptosomes in vitro, similar to the increase in DA clearance observed in striatum in vivo.

Hypothesis #5

The effect of nicotine to increase the Vmax of [³H]DA uptake in striatum is mediated by nicotinic receptors located on DA nerve terminals in striatum.

Hypothesis #6

Systemic administration of nicotine will increase the number of DAT sites in striatum as measured by radioligand binding.

Specific Aim 4

Hypothesis #7

Systemic administration of nicotine will increase the trafficking of DAT to the neuronal cell surface in striatum.

Figure 1. Sagittal slice through a rat brain. Straight lines represent dopaminergic pathways and dotted lines indicate cholinergic projections.

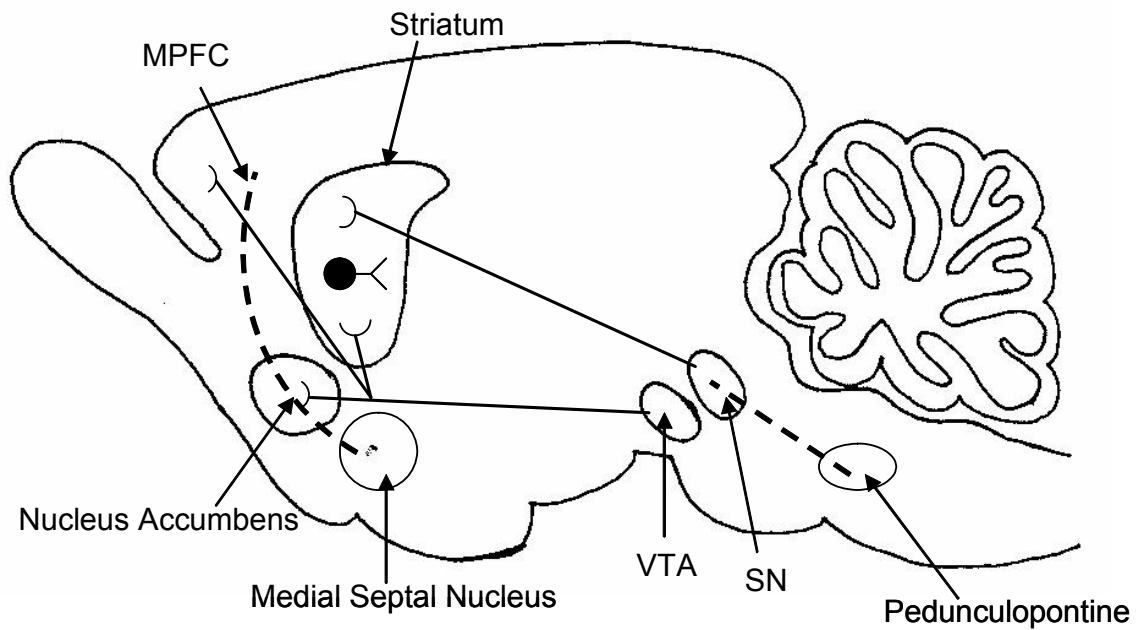
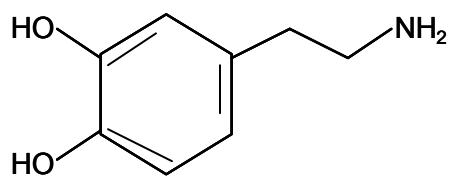
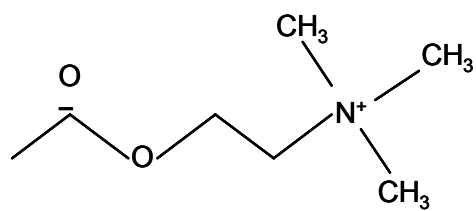


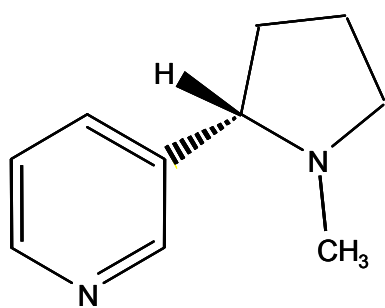
Figure 2. Structures



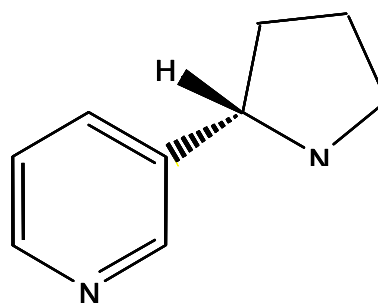
Dopamine



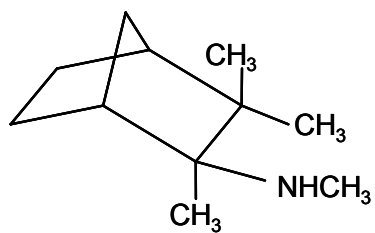
Acetylcholine



Nicotine



Nor nicotine



Mecamylamine

Figure 3. Cartoon representation of a DA nerve terminal. Adapted from Cooper, Bloom and Roth; The Biochemical Basis of Neuropharmacology.

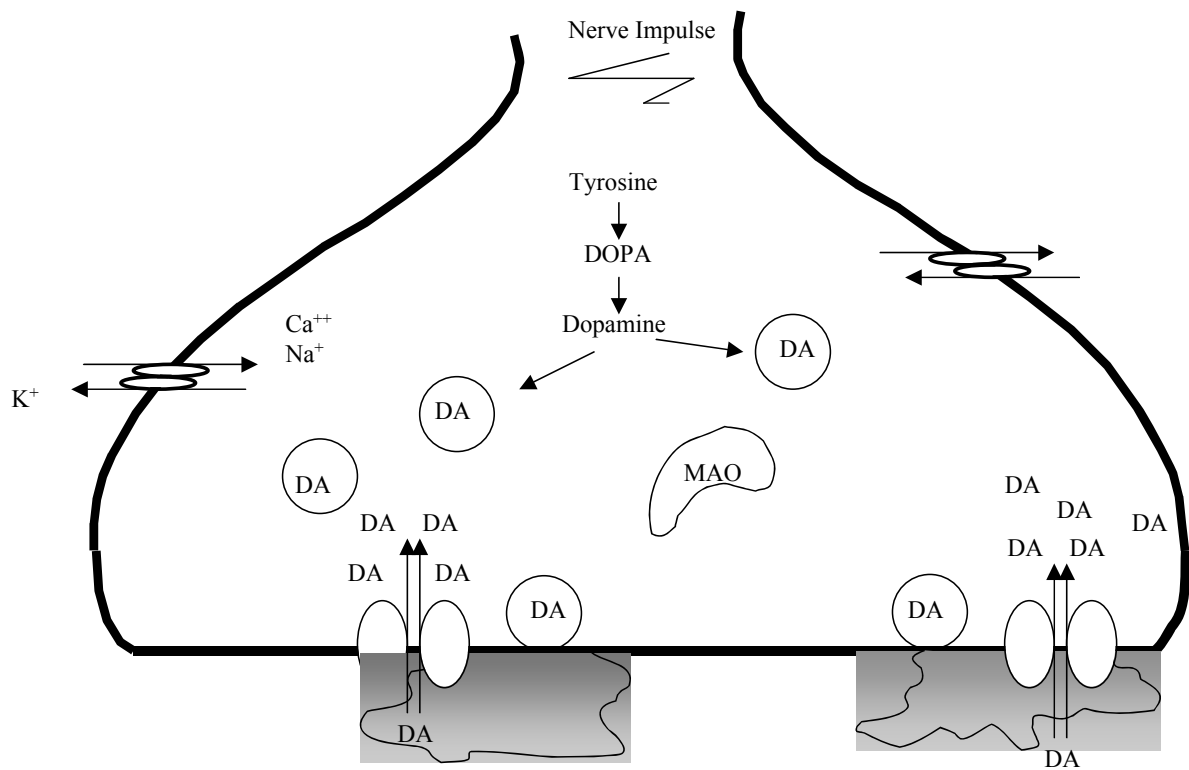


Figure 4. Diagram of a dopamine transporter. DAT has 12 transmembrane domains with intracellular amino and carboxy termini.

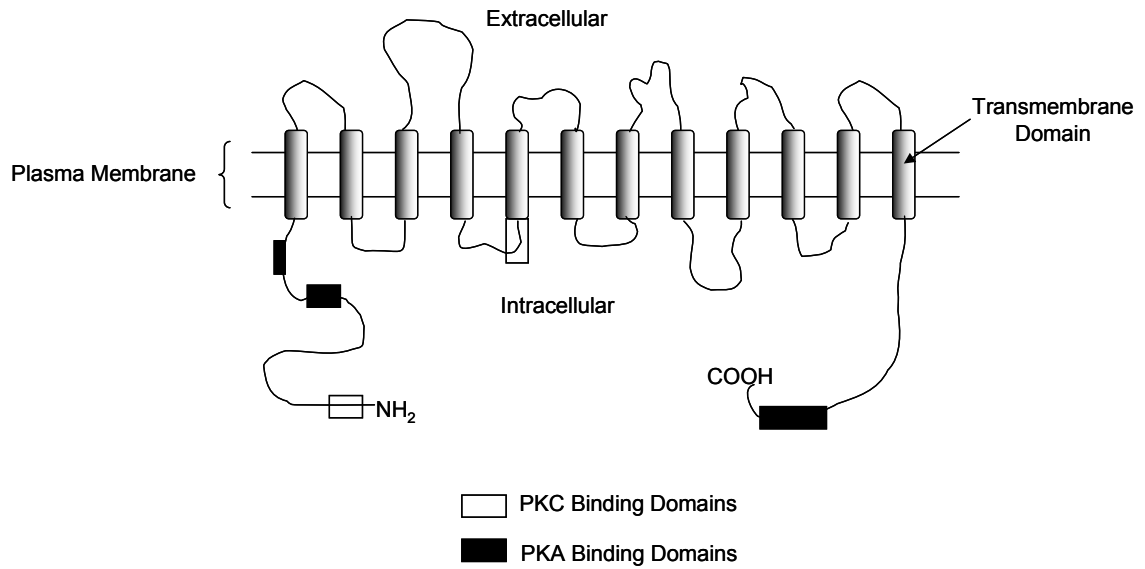
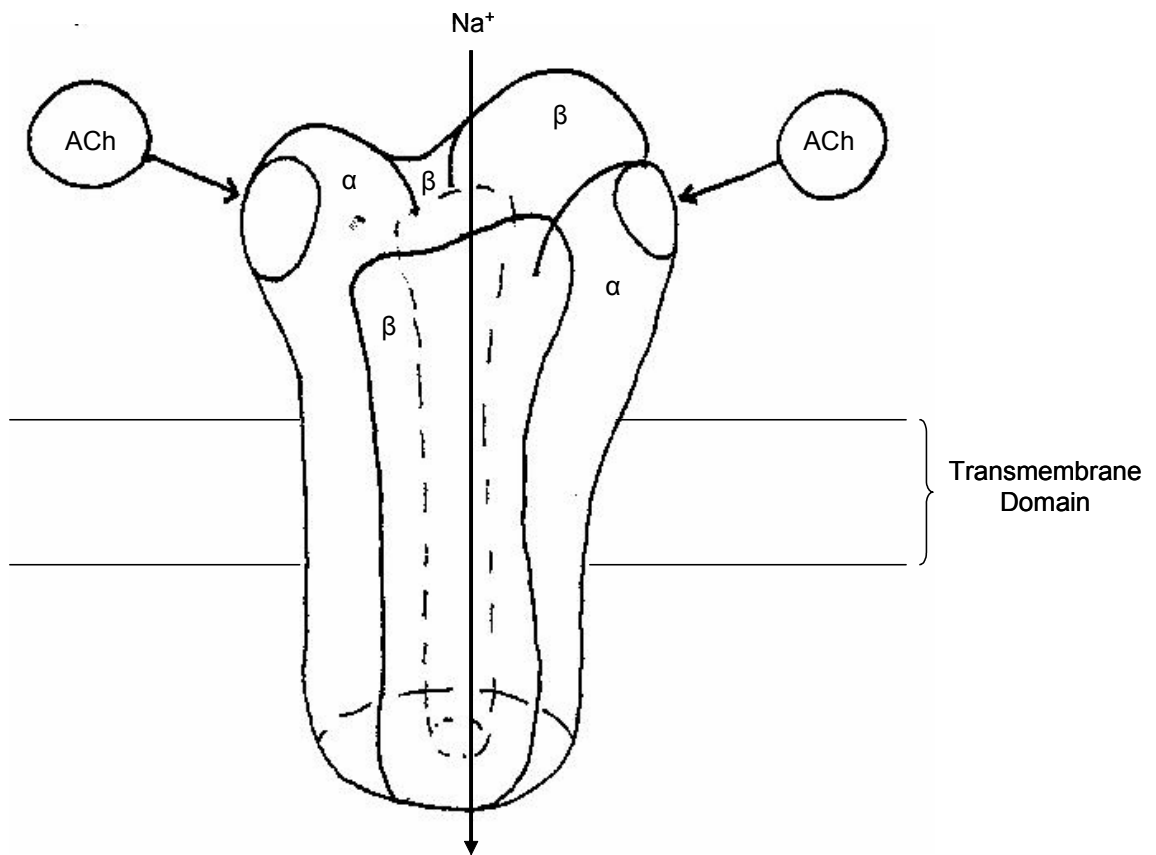


Figure 5. Representation of a neuronal nicotinic acetylcholine receptor.



Chapter Two

Nicotine Increases Dopamine Transporter Function In Vivo in Striatum and Medial Prefrontal Cortex via a Nicotinic Receptor-Mediated Mechanism

I. Introduction

Previous research on the neurobiology of reward and drug addiction has focused on the mesocorticolimbic and nigrostriatal DA systems, emphasizing the role of the nucleus accumbens, MPFC and striatum. The accumbens shell, which is innervated by dopaminergic projections from the ventral tegmental area, and its associated neurocircuitry are believed to encode primary appetitive stimuli associated with unconditioned drug reward (Wise and Bozarth, 1987; Robbins and Everitt, 1996; Bardo, 1998; Koob, 1999; DiChiara, 2000; Kelley and Berridge, 2002). The MPFC, which includes the anterior cingulate cortex and is innervated by dopaminergic projections from the ventral tegmental area, is believed to encode secondary conditioned stimuli associated with environmental cues paired with drug, leading to reward expectancy, which is recognized as important to the process of addiction and relapse to drug use (Berridge and Robinson, 1998; Shima and Tanji, 1998; Kelley, 1999; DiChiara, 2000; Kelley and Berridge, 2002; Peoples, 2002). Integration of the motivational information from the MPFC occurs at least in part in striatal neurons, which are innervated by dopaminergic projections from the nigra, leading to the initiation and execution of movement in reward expectancy and detection of reward (Martin-Soelch et al., 2001).

Nicotine is the most common drug of abuse and tobacco dependence the leading preventable cause of death in the USA (Surgeon General's Report, 1988; Jaffe, 1990). Nicotine, the most abundant alkaloid in tobacco, has intrinsic rewarding properties, which are believed to be responsible for tobacco dependence (Koob, 1992; Corrigall et al., 1992; Stolerman and Jarvis, 1995; Garrett et al., 2003). The mechanisms underlying the reinforcing properties of nicotine are not well understood, although the mesocorticolimbic and nigrostriatal DA systems are believed to be involved. For example, c-fos activation of the anterior cingulate cortex, nucleus accumbens and striatum has been demonstrated in nicotine self-administering rats (Pagliusi et al., 1996; Merlo-Pich et al., 1997), and cortical c-fos has been shown to be activated following exposure to environmental cues associated with repeated nicotine administration (Schroeder et al., 2001). The latter results from animal studies are consistent with findings showing that in tobacco smokers, nicotine dose-dependently increased neuronal activity in the cingulate cortex, frontal lobe and nucleus accumbens, as determined using functional MRI (Stein et al., 1998).

Extracellular DA concentrations are increased following nicotine stimulation of nicotinic acetylcholine receptors in terminal regions of the mesocorticolimbic and nigrostriatal systems. mRNA for nine subunits ($\alpha 2$ – $\alpha 7$ and $\beta 2$ – $\beta 4$) of nicotinic receptors have been identified in substantia nigra and ventral tegmental dopaminergic neurons (Klink et al., 2001; Zoli et al., 2002; Azam et al., 2002; Wooltorton et al., 2003), indicating that potentially a large

number of heteromeric nicotinic receptor subtypes of pentameric structure may be expressed by these neurons. Specific subunit combinations of nicotinic receptors mediating nicotine-evoked DA release (Imperato et al., 1986; Vezina et al., 1992; Nisell et al., 1996, 1997; Teng et al., 1997; Zhou et al., 2001) have not been elucidated conclusively.

Extracellular DA concentrations are the net result of exocytotic DA release from presynaptic terminals and reuptake of DA into presynaptic terminals (clearance) via the DAT. DA is also removed from the extracellular space by metabolism and diffusion, the latter factor playing a larger role in brain regions with sparse dopaminergic innervation and lower DAT density, such as the MPFC. DAT is the major presynaptic terminal protein regulating extracellular DA concentrations and is a presynaptic target for psychostimulant drugs of abuse, as well as for several antidepressant agents. Psychostimulants, such as amphetamine, increase extracellular DA concentrations by reverse transport of DAT (Liang and Rutledge, 1992; Sulzer et al., 1995). Cocaine inhibits DAT function, which results in increased extracellular DA concentrations (Kuhar et al., 1991). The antidepressant and tobacco use cessation agent, bupropion, inhibits DAT function, but also is a nicotinic receptor antagonist (Hurt et al., 1997; Slemmer et al., 2000; Miller et al., 2002). Surprisingly, nicotine (0.4 mg/kg) has been reported to increase DA clearance (enhance DAT function) in the nucleus accumbens in anesthetized rats (Hart and Ksir, 1996). The latter results also contrast findings in vitro in which nicotine and another nicotinic agonist, 1,1-

dimethyl-4-phenyl-piperazinium, have been reported to decrease [^3H]DA uptake into chopped striatum and PC12 cells, respectively (Izenwasser et al., 1991; Huang et al., 1999). However, these in vitro findings have not been replicated (Kramer et al., 1989; Carr et al., 1989; Rowell and Hill 1993; Zhu et al., 2003).

The observation that nicotine enhances DAT function in nucleus accumbens in vivo (Hart and Ksir, 1996) is not expected considering the electrogenic nature of the transporter. Translocation of DA by DAT across the membrane is coupled to the cotransport of two Na^+ ions and one Cl^- ion down their electrochemical gradients, generating a small inward current (Kanner and Schuldiner, 1987; Rudnick and Clark, 1993). Moreover, the DA neuron membrane potential influences DAT function, such that hyperpolarization increases the velocity of DA transport by DAT and depolarization decreases DA transport velocity (Sonders et al., 1997). As such, nicotine-induced stimulation of nicotinic receptors, which results in depolarization of the plasma membrane (Calabresi et al., 1989), would be expected to decrease DAT function and decrease DA clearance, thereby increasing extracellular DA concentrations.

With respect to nicotinic receptor modulation of DAT function, nicotine has also been reported to enhance amphetamine-evoked [^3H]DA release from MPFC slices incubated in assay buffer in the absence of calcium (Drew et al., 2000). These assay conditions precluded nicotine-evoked exocytotic DA release, suggesting the involvement of DAT in the nicotine-induced augmentation of the

response to amphetamine. Surprisingly, nicotine did not enhance amphetamine-evoked [^3H]DA release in striatum or nucleus accumbens (Drew et al., 2000), indicating that this effect was specific to MPFC. The observed nicotine enhancement of the effect of amphetamine in MPFC was inhibited by nicotinic receptor antagonists, mecamylamine and dihydro- β -erythroidine, but not α -bungarotoxin, indicating that specific nicotinic receptor subtypes are involved. The latter findings suggest that nicotinic receptors may modulate DAT function under physiological conditions, i.e., in the presence of extracellular calcium, at least in MPFC.

The goal of the present study was to test the first hypothesis that nicotine will increase DA clearance in the striatum and MPFC in a dose-dependent manner, and this increase in clearance will be mediated by nicotinic receptors. The dose-response relationships for nicotine enhancement of DAT function were characterized in striatum and MPFC. Furthermore, nicotinic receptor mediation was determined by assessing mecamylamine inhibition of the nicotine effect on DAT function in both striatum and MPFC. Under physiological conditions, clearance of exogenously applied DA was assessed with msec resolution using in vivo voltammetry, a technique which has been previously shown to reliably evaluate DAT function (Cass et al., 1992).

II. Methods

Ila. Materials. S(-)-Nicotine ditartrate (nicotine), 3-hydroxytyramine hydrochloride (dopamine, DA), mecamlamine HCl (mecamlamine) and sodium phosphate dibasic were purchased from Sigma/RBI (Natick, MA). Sodium phosphate monobasic, sodium chloride, ascorbic acid and urethane were purchased from Fisher Scientific (Pittsburgh, PA). Nafion perfluorinated ion-exchange resin was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dental wax was purchased from Patterson Dental Supply, Inc. (Louisville, KY). Dental acrylic was purchased from CMA Microdialysis (Acton, MA). Epoxylite and PX grade Graphpoxy were purchased from Epoxylite Corp. (Irvine, CA) and Dylon Industries, Inc. (Cleveland, OH), respectively. Carbon fibers of 30 μm diameter were purchased from Textron, Inc. (Lowell, MA), and 28 gauge lacquer-coated copper wire was purchased from Radio Shack (Lexington, KY).

Ilb. Animals. Male Sprague Dawley rats (200 – 250 g) were obtained from Harlan Laboratories (Indianapolis, IN) and were housed two per cage with free access to food and water in the Division of Lab Animal Resources at the College of Pharmacy, University of Kentucky. Experimental protocols involving the animals were in strict concordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Ilc. In vivo Electrochemical Measurements. Rats were anesthetized with urethane (1.25 - 1.5 g/kg, i.p.) and placed into a Kopf stereotaxic frame

(David Kopf Instruments, Tujunga, CA). Body temperature was maintained at 37 °C with a heating pad coupled to a rectal thermometer (Harvard Apparatus, Holliston, MA). The scalp was reflected, and a section of the skull and dura overlying the frontal cortex were removed. At a location remote from the recording site, a small hole was drilled in the skull above the posterior cortex for placement of two Ag/AgCl reference electrodes, which were cemented into place with dental acrylic.

Electrochemical electrodes were prepared and electrochemical experiments conducted using previously described methods (Cass et al., 1992; Gratton et al., 1989). Electrochemical recording electrodes contained a single carbon fiber (33 μm diameter) in a pulled glass capillary (4 mm outer diameter, 0.80 mm wall diameter) sealed with Epoxylite. Graphite epoxy resin and a 28 gauge lacquer-coated copper wire were inserted into the glass capillary to establish electrical contact with the carbon fiber. The exposed carbon fiber extended 50-150 μm beyond the tip of the glass capillary. Electrodes for some experiments were purchased from Quanteon (Lexington, KY). To enhance selectivity for DA, the carbon fiber electrode was coated with Nafion polymer (5% solution, 6 - 8 coats) and cured by heating at 250 °C for 5 min. Electrodes were calibrated *in vitro* to determine the sensitivity and selectivity for DA. Calibration curves were generated using a range of DA concentrations (1.0 – 10 μM , at 22 °C in 0.1 M PBS solution, pH 4.0). Electrodes showed good sensitivity to DA and were relatively insensitive to ascorbic acid, such that the mean selectivity ratio of

DA/ascorbic acid was 594 ± 57 to 1 ($n = 108$). Subsequently, each carbon fiber electrode was attached to a single barrel micropipette (tip diameter of 10-15 μm) with dental wax. The tips of the electrode and micropipette were positioned 250 - 300 μm apart. The electrode micropipette assembly is shown in Figure 6.

Micropipettes were prepared from monofilament glass (1.0 mm O.D., 0.58 mm I.D.) using a vertical pipette puller (Model 720, David Kopf Instruments, Tujunga, CA). Single barreled micropipettes were filled with DA (200 μM , in 154 mM NaCl and 100 μM ascorbic acid, pH 7.4) immediately prior to conducting the experiment. The concentration of DA for ejection was chosen based on the linear kinetics of DA uptake at this concentration, i.e., DA concentrations that do not saturate DAT in striatum (Zahniser et al., 1999).

The electrode/micropipette assembly was lowered into the dorsal striatum (1.5 mm anterior to bregma, 2.3 mm lateral from midline, and 4.0 - 5.5 mm below the surface of the cortex) or MPFC (cingulate cortex; 2.9 mm anterior to bregma, 1.0 mm lateral from midline, and 2.5 – 5.0 mm below the surface of the cortex), according to the rat brain atlas of Paxinos and Watson (1986). Exogenous DA was pressure ejected (30 - 50 psi, 0.05 ms – 2.5 s) at 5-min intervals using a Picosprizter II (General Valve Corporation, Fairfield, NJ), until reproducible baseline signals (variation in signal amplitude $< \pm 10\%$) were obtained. Using a stereomicroscope fitted with a reticule in one eyepiece, ejection volume (250 nl/mm) was monitored by measurement of the fluid displaced from the micropipette.

In the first series of experiments, dose-response curves for nicotine modulation of DAT function in striatum and MPFC were generated, with nicotine dose and brain region as between-groups factors. Rats were randomly assigned to 5 treatment groups to assess the effect of nicotine (0.1 - 0.8 mg/kg, s.c.) or vehicle (saline) on DAT function in striatum. Rats were assigned to 6 treatment groups to assess the effect of nicotine (0.1 - 0.8 mg/kg, s.c.) or vehicle (saline) on DAT function in MPFC. In an attempt to generate the full dose-response relationship in MPFC, the effect of an additional nicotine dose (0.3 mg/kg) was determined based on the dose-response curve obtained.

For each experiment, nicotine was administered once reproducible baseline signals were obtained. Pressure ejection of DA continued at 5-min intervals for 60 min following nicotine or vehicle injection. In the majority of MPFC experiments, rats were pretreated with desipramine (25 mg/kg, s.c.) to assure that DAT function was measured and that the norepinephrine transporter (NET) did not play a prominent role. Previous in vivo voltammetry studies have shown that the MPFC is heterogeneous with respect to transporter regulation of extracellular DA concentrations (Cass and Gerhardt, 1995). Results from the latter study reveal that at deep recording depths (2.5 – 5.0 mm below the cortical surface), DAT primarily clears DA from the extracellular compartment. At more superficial recording depths (0.5 - 2.25 mm the cortical surface), NET plays a greater role. As such, local application of desipramine into MPFC at superficial

depths resulted in inhibition of DA clearance. At deep recording depths, desipramine had no effect on DA clearance. In contrast, local application of GBR 12909, a selective DAT inhibitor, produced the reverse effect, i.e., inhibited DA clearance at deep recording depths and had no effect at superficial depths in MPFC. In the current study, recording was deeper in the MPFC (2.5 – 5.0 mm below the cortical surface), in an attempt to also ensure that the effect of nicotine on DAT function was evaluated. Comparisons of results in the absence and presence of desipramine revealed no significant differences in the effect of nicotine on DA clearance.

To determine if nicotinic receptors mediated the response to nicotine on DA clearance in striatum and MPFC, the effect of mecamylamine, a noncompetitive nicotinic receptor antagonist (Varanda et al., 1985; Loiacono et al., 1993; Peng et al., 1994), was determined. Rats were randomly assigned to 8 treatment groups (4 treatment groups/brain region). Each group was injected s.c. with either mecamylamine or saline, followed 40 min later by a second injection s.c. of nicotine or saline; such that herein, treatment groups are designated as first/second injection (i.e., mecamylamine/saline, mecamylamine/nicotine, saline/nicotine and saline/saline groups). Once reproducible baseline signals were obtained, mecamylamine (1.5 mg/kg) or vehicle (saline) was injected. The dose of mecamylamine was chosen primarily based on previous behavioral studies from our laboratory and others in which a similar dose of mecamylamine (1.2 mg/kg) inhibited both the acute effects of nicotine on locomotor activity and

the sensitization induced by repeated administration of nicotine (Miller et al., 2001). A lower dose of mecamylamine (1.0 mg/kg) was previously shown to inhibit nicotine-induced locomotor stimulation (Clarke and Kumar, 1983). The dose of mecamylamine chosen was also within the range of that inhibiting nicotine self-administration in rats (Rauhut et al., 2002; Rezvani et al., 2002). Mecamylamine (1.0 mg/kg) also was shown to inhibit the effect of nicotine (0.4 mg/kg) to enhance DA clearance in nucleus accumbens using in vivo voltammetry (Hart and Ksir, 1996). Previous studies have shown that injection of mecamylamine 20 min prior to nicotine injection completely inhibited the nicotine-induced increase in locomotor activity for one hour following nicotine injection (Miller et al., 2001). In the current studies, mecamylamine was injected 40 min prior to nicotine to ensure that mecamylamine had sufficient time to get to the brain and to its site of action. Based on results of the current nicotine dose-response curves in the first series of experiments, nicotine doses (0.8 and 0.4 mg/kg) that produced a maximal effect in striatum and MPFC, respectively, were chosen. DA was pressure ejected every 5 min during the 40 min period following the first s.c. injection and for 60 min following the second s.c. injection. For all experiments, drug dose represents the salt weight, and the injection volume was 1 ml/kg body weight.

High-speed chronoamperometric electrochemical measurements were made continuously (5 times/s, 5 Hz and averaged to 1 Hz), using an electrochemical recording system (IVEC 10; Medical Systems Corporation, Greenvale, NY). The

oxidation potential was a square wave of +0.55 volts applied for 100 ms (versus the Ag/AgCl reference electrode), and the resting potential was 0.0 volts for 100 ms. When the voltage was applied, DA was oxidized to para-ortho-quinone DA resulting in the release of 2 electrons (Figure 6). The electrons coming from the oxidized DA were measured by the electrode and converted to a concentration measure using the IVEC 10. Oxidation currents were digitally integrated during the last 80 ms of each 100-ms pulse.

IId. Data and Statistical Analysis. Data are represented as mean values \pm S.E.M., and n represents the number of animals in a treatment group. Three parameters were determined from the DA oxidation currents; maximal signal amplitude, which is defined by the maximal change in extracellular DA concentration; T_{80} , the time for the signal to decay by 80% minus the rise time of the signal; and DA clearance rate, the slope of the initial pseudolinear portion of the decaying signal (between the T_{20} and T_{60} time points; Figure 7), integrating signal amplitude and time course of decay. During the conduct of this dissertation project, attention was brought to the measure of the clearance rate parameter in that the presentation of the data was backwards from the result of the experiments. Therefore, to alleviate confusion the classical pharmacokinetic measure was utilized (discussed in Chapter Three).

For the first series of experiments generating nicotine dose-response curves in striatum and MPFC, time courses for the nicotine dose effect were

analyzed by two-way mixed-factor ANOVA, with dose as a between-groups factor and time as within-subjects factor. A separate ANOVA was performed to analyze the time course for each of the three parameters assessed, maximal signal amplitude, T_{80} and clearance rate for each brain region. To determine the dose-related effect of nicotine, one-way ANOVAs were conducted on the signal amplitude data from striatum at each 5-min time point during the 30-60 min period following nicotine injection. One-way ANOVAs were also conducted on the dose response for signal amplitude and clearance rate data from MPFC at each time point during the 30-60 min period following nicotine injection. The time period chosen for the latter analyses was based on previous results in rat nucleus accumbens following a single dose (0.4 mg/kg, s.c.) of nicotine (Hart and Ksir, 1996). Dunnett's post hoc analysis was performed to determine differences from saline control. Additionally, specific within-subject contrasts determined the time point at which nicotine significantly decreased signal amplitude compared to baseline (prior to drug injection). Furthermore, for both striatum and MPFC, signal amplitude data were summed across the 60-min recording period to assess nicotine dose-response relationships, and these data were analyzed by one-way ANOVA and Tukey test to determine the nicotine dose that significantly decreased signal amplitude.

Analysis of mecamylamine-induced inhibition of the effect of nicotine on each of the three parameters (maximal signal amplitude, T_{80} and clearance rate) for each brain region was accomplished using separate three-way mixed-factor

ANOVAs, with mecamylamine and nicotine as between-group factors and time as a within-subjects factor. Significant interactions were found for the data expressed as signal amplitude and clearance rate, such that one-way ANOVAs were utilized to assess the effect of nicotine and mecamylamine at individual time points. Additionally, specific within-subjects contrasts were performed for the group administered saline followed by nicotine to determine the time point at which nicotine significantly decreased signal amplitude compared to baseline.

ANOVA, specific contrasts and post hoc analyses were performed using SPSS (standard version 11.0, Chicago, IL). $p < 0.05$ was considered significant. Based on the apriori directional hypothesis being tested, one-tailed tests were considered statistically significant; however, two-tailed tests were reported herein unless otherwise indicated. Nonlinear curve fitting of the cumulative nicotine dose-response data was performed by Prism through a nonweighted iterative process (Prism v3.0, GraphPad software, Inc., San Diego, CA).

III. Results

IIIa. Effect of Nicotine on Exogenous DA Clearance in Striatum. The effect of systemic administration of nicotine (0.1 – 0.8 mg/kg) on exogenous DA clearance in the medial dorsal striatum of urethane-anesthetized rats was determined. Pressure ejection of 200 μ M DA every 5 min resulted in stable baseline signals, with a maximal signal amplitude of 4.21 μ M (\pm 0.22 μ M; mean \pm S.E.M.; range, 1.51 – 9.90 μ M), T_{80} value of 32.6 s (\pm 3.1 s) and clearance rate of

0.307 $\mu\text{M/s}$ ($\pm 0.056 \mu\text{M/s}$; $n = 32$ independent experiments). Maximal signal amplitude during measurement of basal DA clearance varied with the placement of the electrode/micropipette assembly in the striatum. While there was some between group variability in maximal signal amplitude, the within animal baseline signals were stable. Figure 7 illustrates the reproducible pattern of a series of baseline signals for a representative rat obtained prior to systemic injection of nicotine (0.8 mg/kg) or saline (Figure 7, top panel). Maximal signal amplitude under basal conditions for the represented rat was 3.64 μM , which was near the median response and within one SD of the mean response of the group. Once baseline signals had stabilized, rats were injected with either nicotine or saline (control), and pressure ejection of DA continued every 5 min for 60 min. The effect of nicotine on maximal signal amplitude, T_{80} and clearance rate was determined at 5 min intervals and compared to that after saline injection. An overlay of representative signals, obtained in striatum at the 45-min time point after injection when the maximal effect of nicotine (0.8 mg/kg) was observed, reveals a large decrease (45%) in maximal signal amplitude (Figure 7, bottom panel). Rise time, duration and decay of the DA signal were not different between nicotine- and saline-treated rats.

Analysis of the data from the control group (saline injected) reveals that DA signal amplitude gradually decreased by $\sim 15\%$ of baseline during the 55-min period after saline injection; however, a decrease ($\sim 23\%$) in signal amplitude was significant only at the 60-min time point (Figure 8, top panel). Following repeated

DA pressure ejection in the saline control rats, DA signal amplitude decreases and DA clearance increases slightly across the 60-min recording session. It is possible that the decrease in signal amplitude is due to changes in the properties of the recording electrode. During the experiment the electrodes become less sensitive to DA (30% decrease in sensitivity). Therefore, the decrease in signal amplitude may be due to at least in part a decrease in sensitivity of the electrode. While the electrodes are less sensitive, the electrodes retain selectivity for DA. It is also possible that following repeated DA pressure ejection the extracellular space around the ejection site is altered. Thus, changes in extracellular space could modulate DA clearance and play a role in the slight decrease decrease in signal amplitude observed in the saline control group. T_{80} did not change across the time course of the experiment in the saline-injected group (Figure 8, middle panel). Similar to signal amplitude, DA clearance rate also gradually decreased by ~15% of baseline across the time course of the session (Figure 8, bottom panel). The bottom panel illustrates that at the 60-min time point, DA clearance rate decreased by 40%; however, there were no significant main effects or interactions, such that posthoc analyses were not performed. Thus, repeated DA application at 5-min intervals following saline injection resulted in a small increase in DAT function across the 60-min session.

The dose response for nicotine-induced modulation of DAT function in striatum was determined and the results are shown in Figure 8. The parameters of DAT function (maximal signal amplitude, T_{80} and clearance rate) were

analyzed using separate two-way ANOVAs. With respect to signal amplitude, a significant interaction of dose and time was found ($F_{52,351} = 1.58$, $p < 0.05$). To further analyze the interaction, separate one-way ANOVAs of signal amplitude were conducted on the data from each 5-min time point during the 30-60 min after injection (Figure 8, top panel). Dose-related decreases in signal amplitude were observed at various time points. Significant main effects of nicotine dose were found at 40-45 min ($p < 0.05$), and at the 35 min and 50-55 min time points ($p < 0.05$, one-tailed) after injection. Dunnett's post hoc analysis revealed that DA signal amplitude was decreased 35-55 min in the 0.8 mg/kg group compared to the control group. Signal amplitude decreased by a maximum of 48% of control at 45 min after the 0.8 mg/kg dose of nicotine (between-groups comparison); however, when signal amplitude at this time point was compared to the baseline response (within-subject comparison), a 60% decrease in signal amplitude was found. To determine the onset of the effect of nicotine, specific within-subjects contrasts were performed comparing DA signal amplitude at each time point following nicotine injection compared to signal amplitude at baseline. A significant decrease in signal amplitude occurred 15 min following nicotine injection (0.8 mg/kg, $p < 0.01$). In contrast to the effect of nicotine on signal amplitude, no significant main effects or interactions were found for T_{80} or clearance rate, when the data were analyzed by two-way ANOVA (Fig 8, middle and bottom panels, respectively). With respect to clearance rate, the highest dose (0.8 mg/kg) of nicotine tended to increase clearance rate, but did not reach significance. Thus, compared to T_{80} and clearance rate, DA signal amplitude is

the more sensitive parameter for detection of the effect of nicotine on DAT function.

To further evaluate the nicotine dose-response relationship, DA signal amplitude data for each dose were cumulated across the 60-min sampling period to generate a dose-response curve (Figure 9). The nicotine dose-response curve was monophasic, and nonlinear regression revealed a significant fit to the data ($r^2 = 0.99$). Thus, as the nicotine dose increased, DA signal amplitude decreased, indicating that in a dose-related fashion nicotine enhances DAT function in striatum.

IIIb. Effect of Nicotine on Exogenous DA Clearance in MPFC. To assess the effect of nicotine on DAT function in the mesocorticolimbic system, a range of nicotine doses (0.1 – 0.8 mg/kg) was administered s.c. to separate groups of rats, and exogenous DA clearance in MPFC was determined. Pressure ejection of DA (200 μ M) resulted in stable baseline signals with a maximal signal amplitude of 4.54 μ M (\pm 0.18 μ M; mean \pm S.E.M), T_{80} value of 76.8 s (\pm 4.62 s), and clearance rate of 0.085 μ M/s (\pm 0.011 μ M/s; $n = 33$ independent experiments). Once baseline signals had stabilized, groups of rats were injected with either a dose of nicotine (0.1 – 0.8 mg/kg, s.c.) or saline and DA pressure ejection continued at 5-min intervals for 60 min. The effect of nicotine on maximal signal amplitude, T_{80} and clearance rate was determined at 5-min intervals and compared to the saline-injected control group (Figure 10).

Analysis of the data from the saline-injected control group revealed that DA signal amplitude gradually decreased by ~13% during the first 55 min of the session (Figure 10, top panel). At 60 min after the injection of saline, signal amplitude was significantly decreased by 15% ($p < 0.001$). T_{80} was not different from baseline across the duration of the session (Figure 10, middle panel). DA clearance rate tended to decrease (~15% of baseline) across the session time course, but did not reach significance (Figure 10, bottom panel). Thus, under control conditions, repeated DA application at 5-min intervals resulted in a small increase in DAT function across the 60-min session, similar to that observed in striatum.

When the effect of nicotine on both signal amplitude and clearance rate were analyzed by two-way ANOVA, significant interactions of dose and time were found ($F_{65,351} = 1.95$, $p < 0.001$ and $F_{65,351} = 1.74$, $p < 0.01$, respectively). No significant main effects or interactions were observed for T_{80} . Signal amplitude data were analyzed further by one-way ANOVAs at individual 5-min time points, revealing dose-related decreases in signal amplitude at 15-30 min and 50-60 min ($p < 0.05$; at 35 min, $p < 0.05$, one-tailed, Figure 10, top panel). Dunnett's post hoc analysis revealed that compared to the control group, DA signal amplitude was decreased in the 0.4 mg/kg group at 15-30 min ($p < 0.05$), and at the 35 min and 50-55 min time points ($p < 0.05$, one-tailed). Compared with the control group, a maximal decrease of 46% in signal amplitude was

observed 30 min after injection of nicotine (0.4 mg/kg); and when the data at this time point were compared to the within-subject baseline, a 53% decrease in signal amplitude was observed. To assess the onset of nicotine effect, specific within-subjects contrasts were performed comparing signal amplitude at each time point after injection to that at baseline. A significant ($p < 0.001$) decrease in signal amplitude was observed 10 min following nicotine (0.4 mg/kg) injection.

With respect to clearance rate, one-way ANOVAs were conducted to assess the nicotine dose-response at each 5-min time point after injection (Figure 10, bottom panel). Dose-related decreases in clearance rate were observed at 15-25 min and 60 min ($p < 0.05$), and at the 30 min and 50-55 min time points ($p < 0.05$, one-tailed) post injection. Dunnett's post hoc analysis revealed that DA clearance rate was decreased in the 0.4 mg/kg group compared to the control group at 15-25 min ($p < 0.05$) and at 30 min ($p < 0.05$, one-tailed) after injection. Compared to the control, clearance rate after 0.4 mg/kg decreased by a maximum of 33% at the 45-min time point; whereas a maximum decrease of 44% was observed when compared to the within-subject baseline response. The onset of the nicotine effect on clearance rate occurred at 10 min following nicotine injection (0.4 mg/kg, $p < 0.01$).

DA signal amplitude was also expressed as a cumulative change across the 60-min sampling period to evaluate the nicotine dose-response relationship (Figure 11). Surprisingly, a U-shaped dose-response relationship was apparent.

Only the 0.4 mg/kg dose of nicotine decreased DA signal amplitude in MPFC. Similarly, a U-shaped dose-response relationship was observed for clearance rate (data not shown). Thus, nicotine also modulates DAT function in MPFC; however, the dose-response pattern was different from that observed in striatum, and the time of onset and of maximal response occurred more rapidly after systemic nicotine injection in MPFC than in striatum.

IIIC. Effect of Mecamylamine on Nicotine-induced Modulation of DAT Function in Striatum. To ascertain if the effect of nicotine on DAT function is mediated by nicotinic receptors, the ability of mecamylamine to inhibit the nicotine-induced increase in DA signal amplitude was determined. Pressure ejection of DA (200 μ M) resulted in a maximal signal amplitude of 6.75 μ M (\pm 0.43 μ M; mean \pm S.E.M.), T_{80} value of 23.8 s (\pm 4.60 s), and clearance rate of 0.902 μ M/s (\pm 0.119 μ M/s; n = 26 independent experiments). Once baseline signals stabilized, groups of rats were injected with mecamylamine (1.5 mg/kg, s.c.) or saline, and 40 min later with nicotine (0.8 mg/kg, s.c.) or saline. The dose of nicotine was chosen based on the above dose-response analysis. DA pressure ejection continued every 5 min after mecamylamine or saline and every 5 min for 60 min after nicotine or saline. Data for the three parameters of DA clearance are illustrated in Figure 12.

Since signal amplitude was the only parameter which detected the dose relationship for nicotine to enhance DAT function in striatum, a three-way

ANOVA on signal amplitude data was performed to determine if mecamylamine inhibited the effect of nicotine. A significant three-way interaction of mecamylamine x nicotine x time was found ($F_{13,286} = 1.82, p < 0.05$). As previously observed, DA signal amplitude tended to gradually decrease across the 60-min session when compared to baseline in the Saline-Saline control group; however, only at the 60-min time point did the decrease (25%) in signal amplitude reach significance. In the Saline-Nicotine group, the onset of the effect of nicotine to decrease signal amplitude (30%) occurred at 25 min after nicotine injection (within-subject comparisons, $p < 0.01$). At the 55-min time point, nicotine maximally decreased signal amplitude by 56% compared to baseline. At the latter time point, comparison of the Saline-Nicotine and Saline-Saline groups revealed that nicotine decreased signal amplitude by a maximum of 36%. With respect to the Mecamylamine-Saline group, no within-subject differences were observed across the time course of the session compared to baseline, indicating that mecamylamine alone had no effect on DAT function. To determine if mecamylamine inhibited the effect of nicotine, one-way ANOVAs compared the treatment groups at each time point beginning at the onset of nicotine's effect (i.e., 25-60 min). At the 40, 45, 55 and 60 min time points, signal amplitude in the Mecamylamine-Nicotine group was significantly greater than that in the Saline-Nicotine group ($p < 0.05$, one-tailed) and not different from that in the Saline-Saline group, indicating that mecamylamine completely inhibited the effect of nicotine on DAT function in striatum.

IIId. Effect of Mecamylamine on Nicotine-induced Modulation of DAT

Function in MPFC. To ascertain if nicotinic receptors also mediate nicotine-induced modulation of DAT function in MPFC, the ability of mecamylamine to inhibit the nicotine-induced decrease in signal amplitude in urethane-anesthetized rats was determined. Stable baseline signals, obtained in response to pressure ejection of 200 μ M DA, exhibited a maximal signal amplitude of 4.14 μ M (\pm 0.27 μ M; mean \pm S.E.M.), T_{80} value of 80.5 s (\pm 6.02 s), and clearance rate of 0.066 μ M/s (\pm 0.008 μ M/s; n = 24 independent experiments). Once baseline signals stabilized, experiments were performed as described above, except that electrochemical measurements were made in MPFC (Figure 13). A three-way ANOVA was performed to determine if mecamylamine inhibited the nicotine-induced decrease in DA signal amplitude in MPFC. A significant interaction of mecamylamine x nicotine x time ($F_{13,260} = 2.08$; $p < 0.02$) was found. DA signal amplitude decreased to a maximum of 20% compared to baseline at 60 min following the second saline injection in the Saline-Saline group. Within-subjects comparison in the Saline-Nicotine group revealed that the onset of the nicotine effect occurred 15 min after nicotine injection, at which time a significant ($p < 0.001$) decrease in signal amplitude was observed compared to baseline response. At the 35 min time point, nicotine produced a maximal decrease in signal amplitude (45%) compared to baseline; between-groups comparison of the response in the Saline-Nicotine and Saline-Saline groups revealed a maximum decrease of 25% at this time point. Within-subjects comparisons in the Mecamylamine-Saline group revealed no differences across the session

compared to baseline, indicating that mecamylamine alone had no effect on DAT function in MPFC. One-way ANOVA followed by Dunnett's test compared data from the Mecamylamine-Nicotine and Saline-Nicotine groups at each time point from 15-60 min, when nicotine decreased signal amplitude compared to baseline. Signal amplitude was greater in the Mecamylamine-Nicotine group than that in the Saline-Nicotine group at 20-30, 40 and 50 min ($p < 0.05$) and 35, 45 and 60 min ($p < 0.05$, one-tailed) of the session. Importantly, the response in the Mecamylamine-Nicotine group was not different from that in the Saline-Saline group at these time points, indicating that mecamylamine completely inhibited the effect of nicotine on DAT function in MPFC. Thus, these results suggest that nicotine modulates DAT function *via* a nicotinic receptor-mediated mechanism in MPFC.

IV. Discussion

The results from the current *in vivo* voltammetry study demonstrate that nicotine in a dose-related manner decreases DA signal amplitude in both striatum and MPFC, indicating that nicotine enhances DA clearance in both brain regions. However, across the same nicotine dose range, differential patterns in the nicotine dose-response curve were observed in striatum and MPFC. That is, a monophasic dose-response curve was observed in striatum, whereas a U-shaped curve was found in MPFC, both curves having a maximal 50% effect. Maximal effect occurred at a lower dose in MPFC than in striatum (0.4 and 0.8 mg/kg, respectively). In both brain regions, the onset of a significant effect on DA

clearance occurred 10-15 min after nicotine injection; however, DA clearance tended to increase by 5 min after nicotine injection. Additionally, the time to maximal response was more rapid in MPFC compared to striatum (30 and 45 min, respectively). The time course of the response to nicotine in both brain regions is in good agreement with pharmacokinetic data showing a maximal nicotine concentration in rat brain at 5 min after peripheral nicotine injection and a brain $t_{1/2}$ of 52 min (Ghosheh et al., 2001). Of note, the nicotine-induced increase in DA clearance in striatum lasted for at least 2 hours following nicotine injection. Thus, the effect of nicotine on DAT function is long-lasting. Nevertheless, the pattern of the nicotine dose-response curve in the present study was different between MPFC and striatum, with MPFC showing greater sensitivity to nicotine.

The current results support and extend previous findings, showing that systemically administered nicotine increases DA clearance in several dopaminergic terminal regions, including MPFC and striatum (current study), and nucleus accumbens (Hart and Ksir, 1996). The latter results are surprising since stimulation of nicotinic receptors results in depolarization of the plasma membrane (Calabresi et al., 1989) and since depolarization of the membrane generally decreases DA transport velocity (Sonders et al., 1997). However, this was not the case with nicotine. The current results demonstrate a dose-related nicotine-induced enhancement of DAT function in striatum and MPFC, and extend the findings of Hart and Ksir (1996) in nucleus accumbens.

Interestingly, following nicotine injection, nicotine-induced increases in DA release are not observed using this procedure. It is possible that the electrodes used in the current study are not sensitive enough to be able to detect the released DA. However, a more likely explanation is that the amount of DA released by nicotine at the recording site was not large enough to be detected. Thus, while nicotine has been shown in several studies to release DA, this mechanism does not appear to have an effect on the nicotine-induced increase in DA clearance.

The present study also demonstrates that the nicotine-induced enhancement of DA clearance in both MPFC and striatum was inhibited by pretreatment of the rats with mecamylamine, a nonselective nicotinic receptor antagonist (Varanda et al., 1985). These results suggest that nicotine stimulates nicotinic receptors to increase DAT function in these brain regions. In the current study, mecamylamine had no effect on its own, but inhibited the effect of nicotine on DA clearance in both striatum and MPFC. The current results are in agreement with the work of Hart and Ksir (1996), who also reported no effect of mecamylamine alone on DAT function in nucleus accumbens. The observation that mecamylamine had no effect alone, suggests that nicotinic receptors, which modulate DAT function in these brain regions, are not tonically activated.

The involvement of distinct nicotinic receptor subtypes in striatum and MPFC provides a likely explanation for the differential nicotine dose-response

pattern observed in the current study. Recent studies have demonstrated the presence of multiple mRNAs for nicotinic receptor subunits ($\alpha 2$ - $\alpha 7$ and $\beta 2$ - $\beta 4$) and their respective proteins in DA cell bodies in both substantia nigra and ventral tegmental area; however, differences in the relative abundance of these subunits in substantia nigra and ventral tegmental area have also been reported (Klink et al., 2001; Azam et al., 2002; Zoli et al., 2002; Wooltorton et al., 2003). The nicotinic receptors expressed in the nigrostriatal and mesocorticolimbic DA systems depend on the specific combinations of subunits forming functional nicotinic receptors. Pairwise expression of nicotinic receptor subunits in *Xenopus* oocytes initially revealed characteristic pharmacological profiles, i.e., relative sensitivity and responsiveness to a range of nicotinic ligands (Luetje and Patrick, 1991). Inclusion of a third type of subunit (e.g., $\alpha 5$ with $\alpha 3$ and $\beta 2$ subunits) in similar expression systems further altered the physiological response, calcium permeability and desensitization characteristics of the expressed nicotinic receptor subtypes (Gerzanich et al., 1998). Characterization of the physiological response of individual neurons in midbrain slices revealed four different patterns of nicotinic receptor-mediated currents, revealing the complexity of native nicotinic receptors which purportedly contained as many as four different subunits (Klink et al., 2001).

Although the specific subunit composition of nicotinic receptor modulating DAT function in either striatum or MPFC is not known, the nicotinic receptor subtype modulating DAT function in MPFC may be more sensitive to nicotine

than the specific nicotinic receptor subtype modulating DAT function in striatum, since the nicotine dose producing an enhancement of DA clearance is lower in MPFC than in striatum. The current results show that in both striatum and MPFC, 0.1 and 0.3 mg/kg produce no effect on DAT function; however, 0.4 mg/kg enhances DAT function in MPFC, whereas 0.8 mg/kg enhances DAT function in striatum. Thus, the descending portion of the dose-response in MPFC resembles the dose-response in striatum, but the curve from MPFC is shifted to the left of that from striatum. Doses of nicotine above 0.8 mg/kg were not examined in the current study, since such doses elicit seizures that may have confounded the results. Thus, the differential pattern of response observed in the present study in MPFC and striatum with respect to nicotinic receptor-mediated modulation of DAT function is likely the result of stimulation of distinct nicotinic receptor subtypes.

An alternative explanation for the differential nicotine dose-response relationships in striatum and MPFC is the lower dopaminergic terminal density in MPFC compared to striatum, and the decreased number of DAT per terminal in MPFC compared to striatum (Sesack et al., 1998). It may be that a higher dose of nicotine is required to observe the modulation of DAT function in striatum simply due to the greater number of DAT protein in striatum compared to MPFC.

Another potential explanation for the difference in regional dose-response is that the local neuronal circuitry is different between these two brain regions,

i.e., different afferents impinge on the dopaminergic terminals in striatum and MPFC potentially providing differential regulation of DAT function. In this regard, the U-shaped function in MPFC may be the result of nicotine-induced stimulation of an additional neurotransmitter system in the MPFC. The result of activation of the additional neurotransmitter may have opposed the nicotinic receptor-mediated enhancement of DAT function. For example, high concentrations of nicotine have been shown to activate $\alpha 7$ nicotinic receptors, resulting in glutamate release in frontal cortex (Kaiser and Wonnacott, 2000; Schilstrom et al., 2000; Marchi et al., 2002). For example, stimulation of metabotropic glutamate receptors have been reported to decrease DAT function (Page et al., 2001). Thus, activation of $\alpha 7$ receptors indirectly through glutamate neurotransmission could result in inhibition of DAT function, counteracting activation of the high affinity heteromeric nicotinic receptors, which enhance DAT function.

Alternatively, nicotinic receptors may indirectly modulate DAT function through activation of neural circuitry at the cell body level. Local administration of nicotine into the substantia nigra and ventral tegmental area has been shown to evoke DA release in striatum and nucleus accumbens, respectively, via stimulation of nicotinic receptors in the cell body region (Blaha and Winn, 1993; Sziraki et al., 2002). Additionally, the effect of peripheral administration of nicotine to increase DA release in the nucleus accumbens determined using microdialysis was inhibited by local administration of mecamylamine into the

ventral tegmental area (Sziraki et al., 2002). Thus, it seems plausible that in the current study, peripherally administered nicotine may be acting at nicotinic receptors at the level of the cell body to modulate DAT function at the terminal. Furthermore, different nicotinic receptor subtypes expressed at the cell body may be responsible for the different dose-response patterns observed in MPFC and striatum with respect to DA clearance.

In both striatum and MPFC, the mechanism by which nicotine modulates DAT function may be via nicotinic receptor-induced augmentation of DAT trafficking to the presynaptic terminal membrane consistent with an increase in DA clearance. The relatively rapid nicotine-induced increase in DA clearance suggests that new synthesis of DAT protein is not responsible. Rather, the time course of response is consistent with trafficking of intracellular stores of DAT protein to the terminal membrane. Cocaine, another drug of abuse, has been shown to dynamically regulate DAT function by increasing DAT trafficking to the plasma membrane in cells expressing hDAT (Little et al., 2002). In contrast, amphetamine diminished DAT localization at the plasma membrane in hDAT expressing cells (Saunders et al., 2000) and in rat striatal dopaminergic terminals (Fleckenstein et al., 1997). Investigation of the effect of nicotine on DAT trafficking is warranted based on the enhanced DA clearance using *in vivo* voltammetry in the current study.

Due to the lower DAT density and decreased number of DAT per terminal in MPFC compared to striatum, metabolism and diffusion likely play a larger role in clearing DA from the extracellular space in MPFC under physiological conditions. However, following pharmacological treatment with nicotine, enhanced DAT function in MPFC would be predicted to have a large impact on dopaminergic transmission. Nicotine enhancement of DAT function would result in more efficient DA clearance from the extracellular space, and cortical function would be disinhibited. Thus, the ability of nicotinic receptors to modulate DAT function, and thereby extracellular DA concentration, may have physiological importance with respect to nicotine enhancement of cognitive processes such as attention, learning and memory, as well as important clinical relevance with respect to schizophrenia and drug abuse.

Figure 6. Schematic representation of the electrode micropipette

assembly. A micropipette filled with DA is attached to a nafion-coated electrode.

Following DA pressure ejection, DA is oxidized to para-ortho-quinone DA releasing 2 electrons. The electrons are measured by the electrode and the electrochemical signal is converted to a concentration measurement using the IVEC-10 system allowing for determination of the DA concentration at the electrode site.

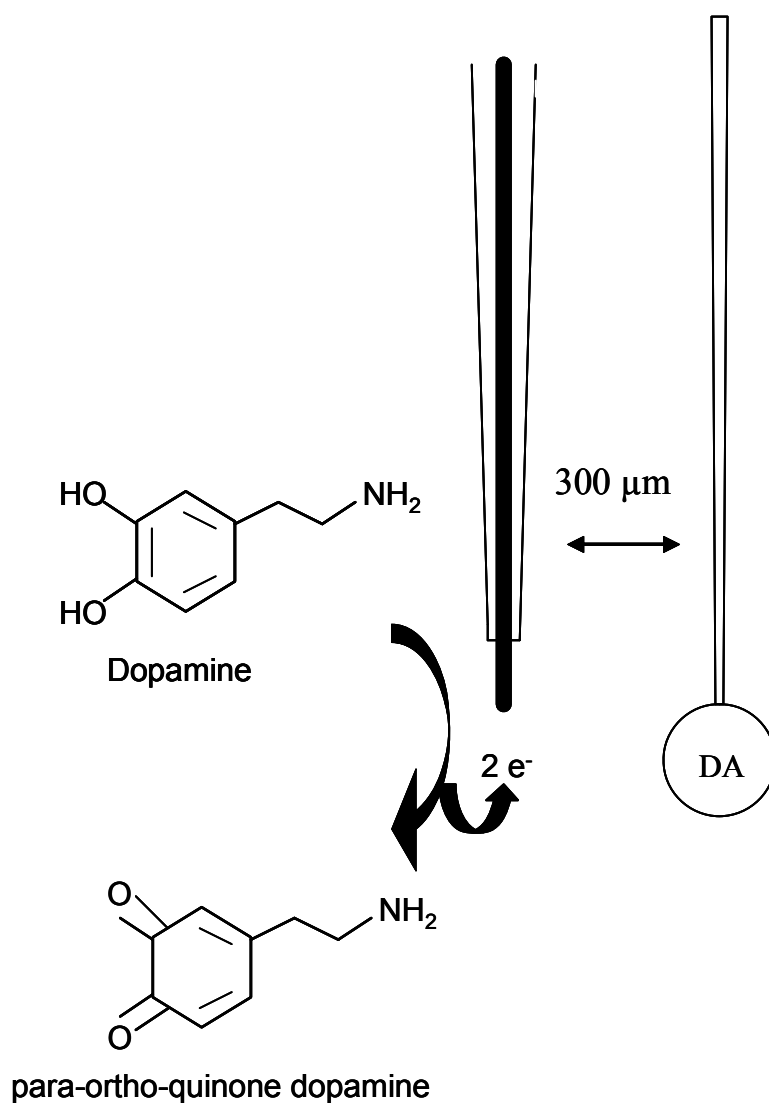


Figure 7. Representative DA signals in striatum of urethane-anesthetized rats prior to (top panel) and 45 min following nicotine or saline injection (bottom panel). Reproducible electrochemical signals were obtained following pressure ejection of DA (200 μ M) and represent the baseline response (top panel). Representative DA signals 45 min following injection of nicotine (NIC; 0.8 mg/kg, s.c.) or saline are represented by dashed and solid lines, respectively (bottom panel). Data are expressed as μ M DA as a function of time (sec) after DA pressure ejection. DA concentrations were calculated based on calibration curves generated in vitro.

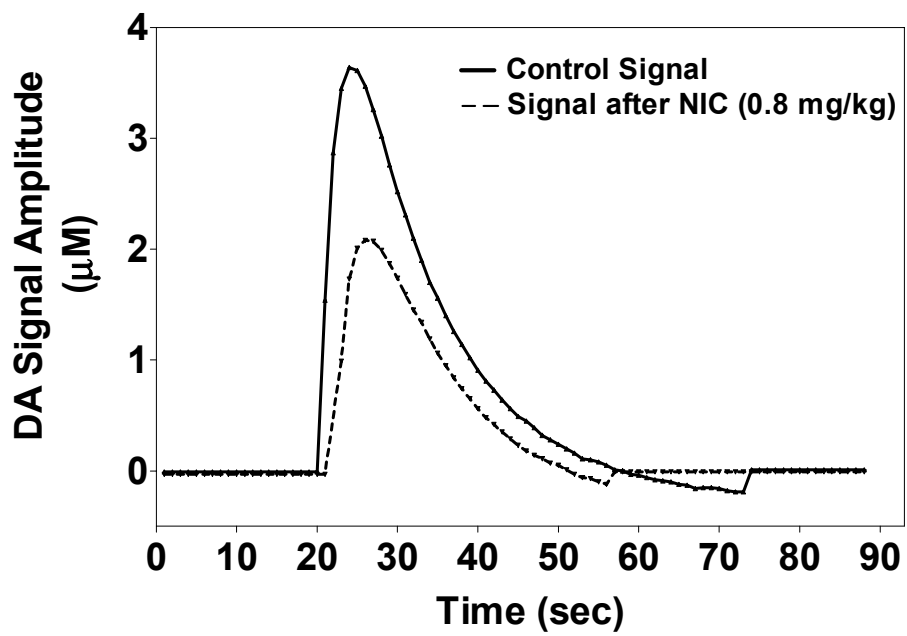
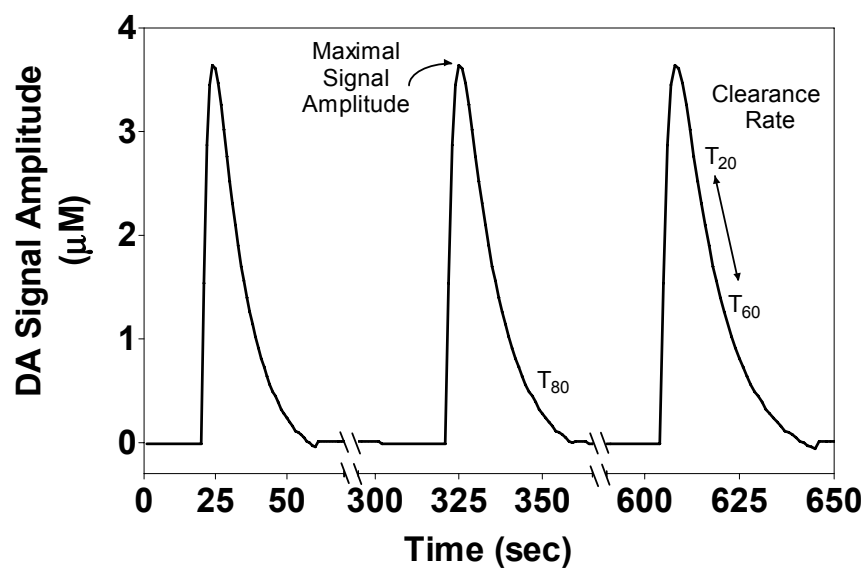


Figure 8. Dose-response for systemically administered nicotine to alter DA signal amplitude (top panel), T_{80} (middle panel), and clearance rate (bottom panel) in striatum of urethane-anesthetized rats. Nicotine in a dose-related manner decreased signal amplitude, but did not significantly alter T_{80} or clearance rate across 60-min recording sessions. After stable baseline signals were obtained in response to pressure ejection of DA (200 μ M) at 5-min intervals, either a dose of nicotine (NIC; 0.1 – 0.8 mg/kg) or saline was injected s.c. (indicated by the arrow), and pressure ejection of DA at 5-min intervals continued for 60 min. Data are expressed as mean, S.E.M. as a percentage of the respective baseline values as a function of time (min). Mean baseline values for each parameter are provided in the Results section. (n = 5 - 8 rats/group)

* difference from saline control at the corresponding time point ($p < 0.05$); + first time point at which nicotine decreased signal amplitude compared to the corresponding basal.

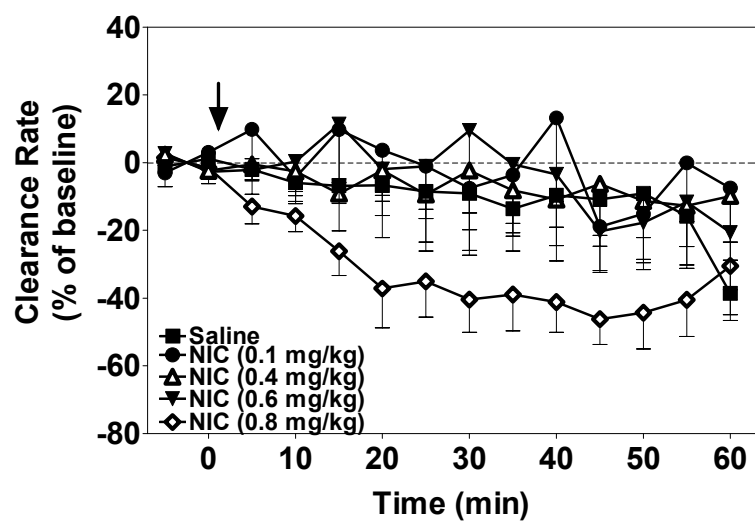
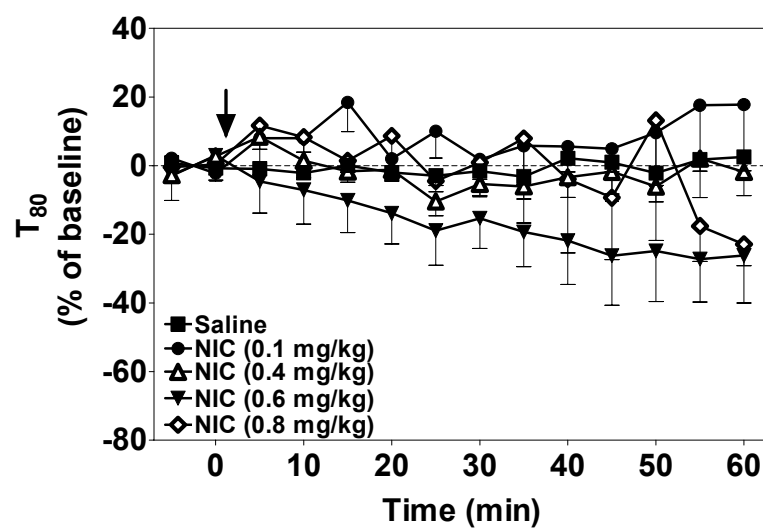
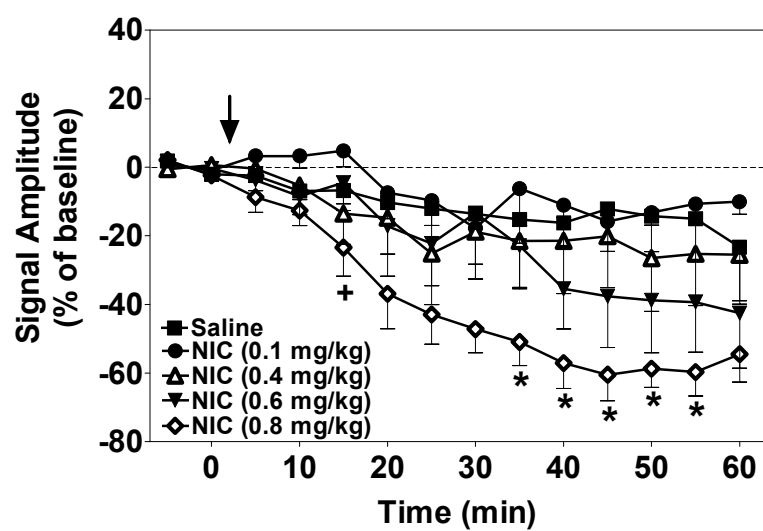


Figure 9. In a dose-related manner, nicotine decreased signal amplitude cumulated across the 60-min sampling period. For each experiment, the change in signal amplitude was cumulated across the 60-min period of electrochemical recording in striatum. Data are expressed as mean \pm S.E.M. as a percent of baseline. (n = 5 – 8 rats/group)

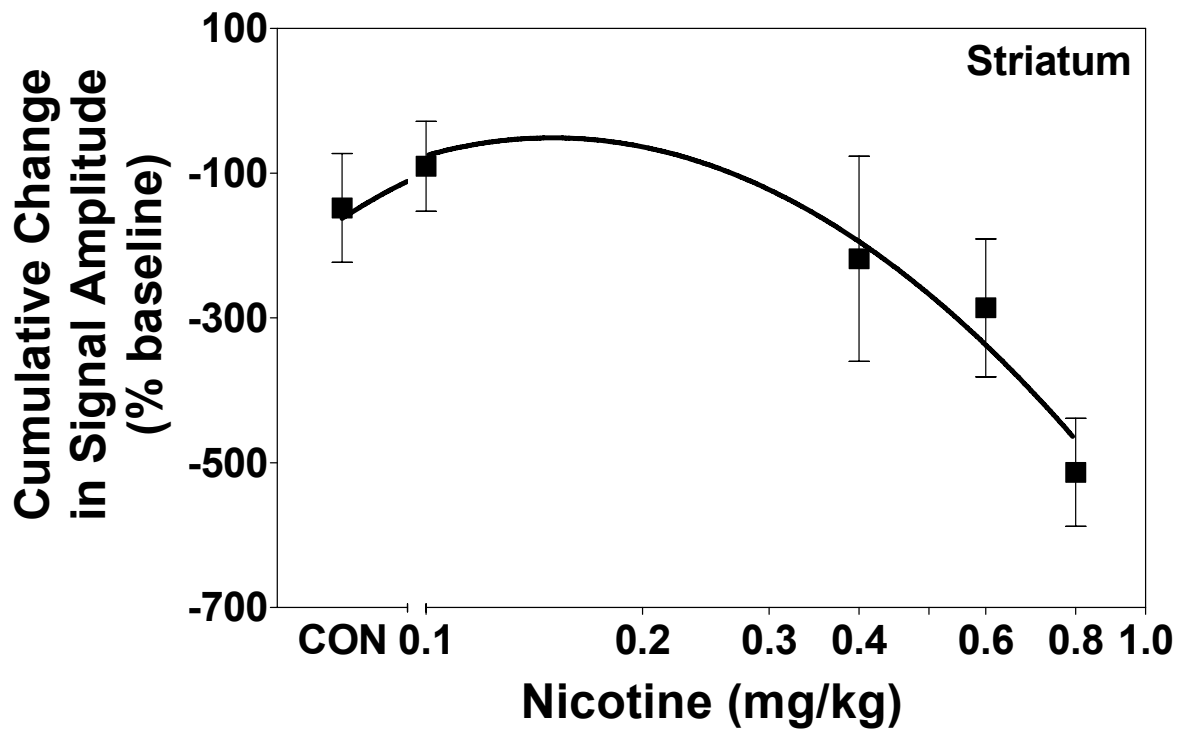


Figure 10. Dose-response for systemically administered nicotine to alter DA signal amplitude (top panel), T_{80} (middle panel) and clearance rate (bottom panel) in MPFC of urethane-anesthetized rats. Nicotine in a dose-related manner decreased signal amplitude (top panel) and DA clearance rate (bottom panel), but did not alter the T_{80} (middle panel) across the 60-min recording session. After stable baseline signals were obtained in response to pressure ejection of DA (200 μ M) at 5-min intervals, either a dose of nicotine (NIC; 0.1 – 0.8 mg/kg) or saline was injected s.c. (indicated by the arrow), and DA pressure ejection continued at 5-min intervals for 60 min. Data are expressed as mean, S.E.M. as a percentage of within-subject baseline values as a function of time (min). Baseline values for each parameter are provided in the Results section. (n = 5 - 7 rats/group) * difference from saline control at the corresponding time point ($p < 0.05$); # difference from saline control at the corresponding time point ($p < 0.05$; one-tailed); + first time point at which nicotine decreased signal amplitude compared to the corresponding basal.

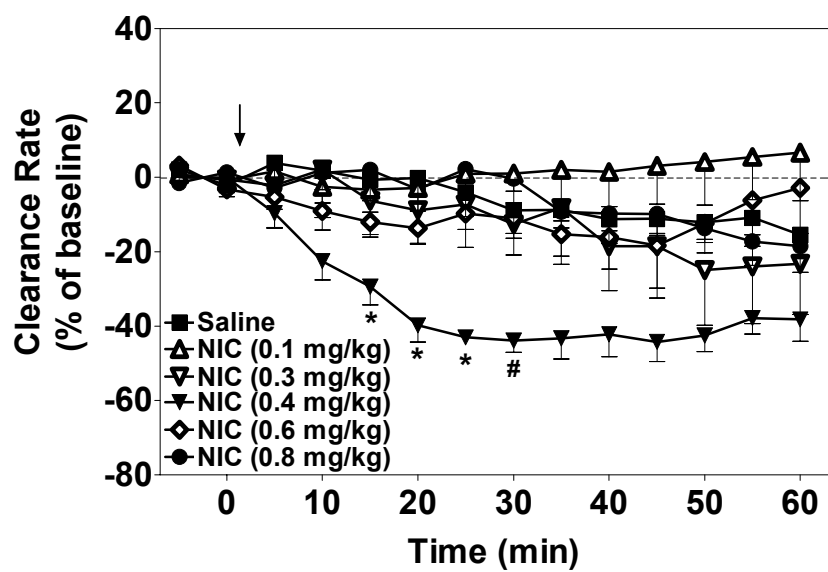
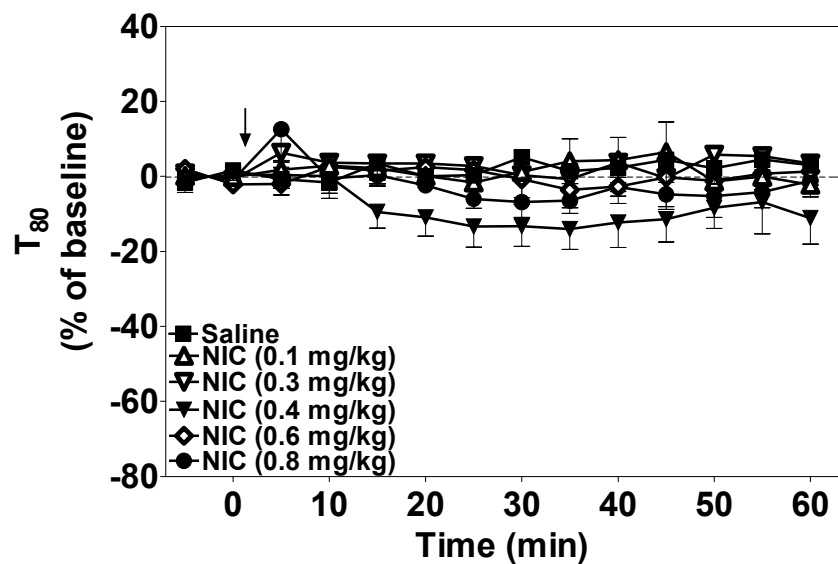
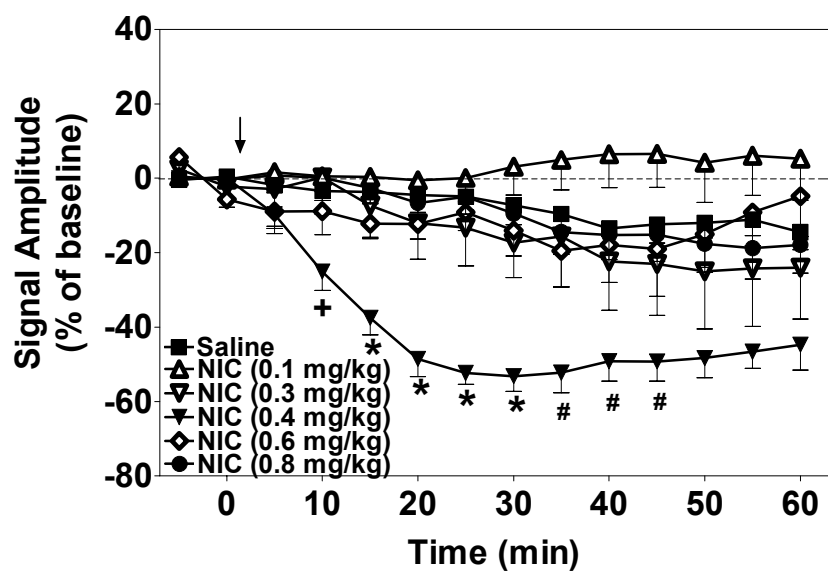


Figure 11. In a dose-related manner, nicotine decreased signal amplitude cumulated across the 60-min sampling period. For each experiment, the change in signal amplitude was cumulated across the 60 min period of electrochemical recording in MPFC. Data are expressed as mean \pm S.E.M. as a percent of baseline. (n = 5 – 7 rats/group)

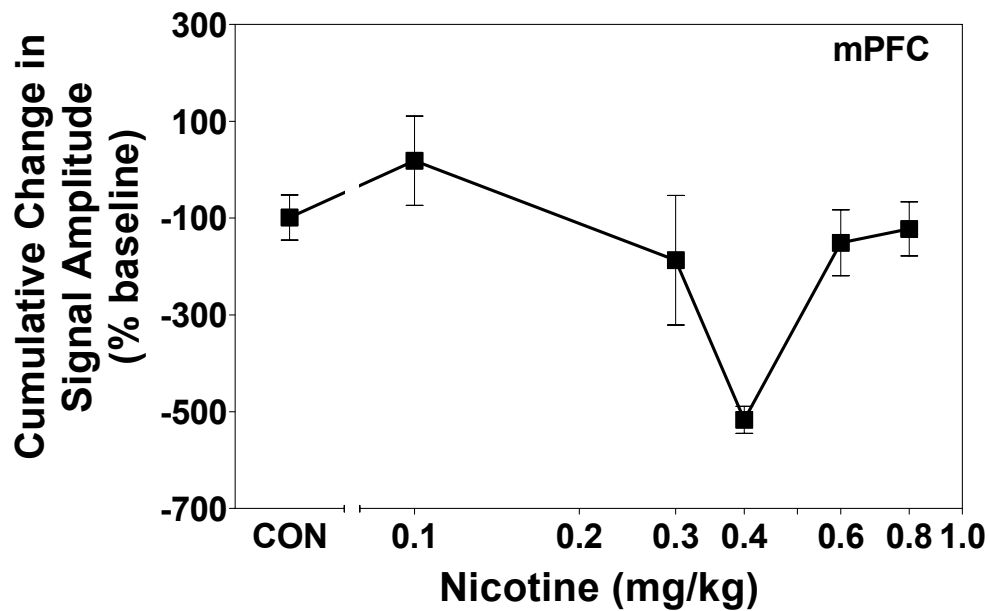


Figure 12. Effect of mecamlamine to inhibit the nicotine-induced decrease in signal amplitude in striatum. Signal amplitude is shown in the top panel; T_{80} and clearance rate are shown in middle and bottom panels, respectively, for comparison. No significant differences in were observed in T_{80} or clearance rate. After stable baseline signals were obtained in response to pressure ejection of DA (200 μ M) at 5-min intervals, either mecamlamine (MEC; 1.5 mg/kg) or saline was injected s.c. and 40 min later nicotine (NIC; 0.8 mg/kg) or saline was injected s.c.(second injection indicated by the arrow). DA continued to be pressure ejected at 5-min intervals for 60 min following the second injection. Data are expressed as mean, S.E.M. as a percentage of the respective baseline values as a function of time (min). Baseline values for each parameter are indicated in the Results section. Legend indicates treatment group designating first and second injections. (n = 6 - 7 rats/group) [#] difference between Mecamlamine/Nicotine group and Saline/Nicotine group at the corresponding time point ($p < 0.05$; one-tailed); ⁺ first time point at which nicotine decreased signal amplitude compared to the corresponding basal.

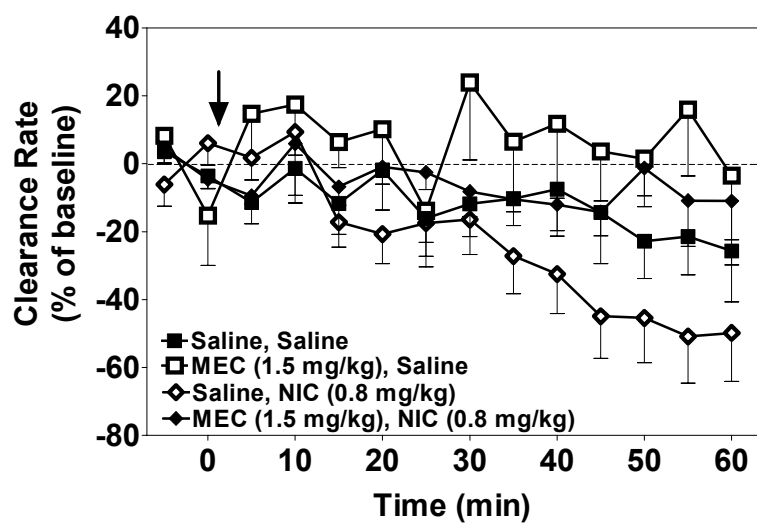
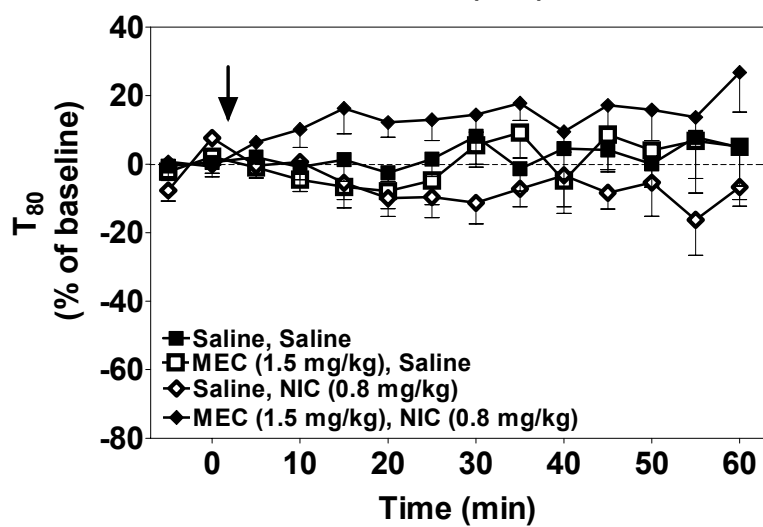
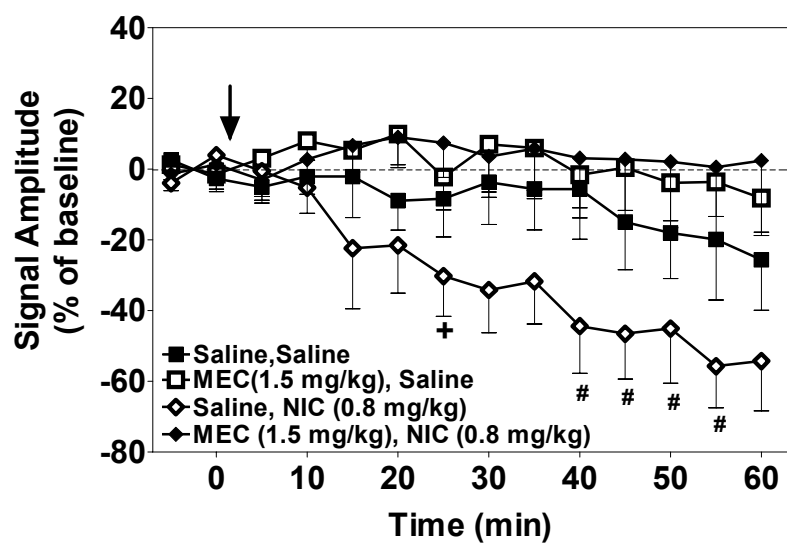
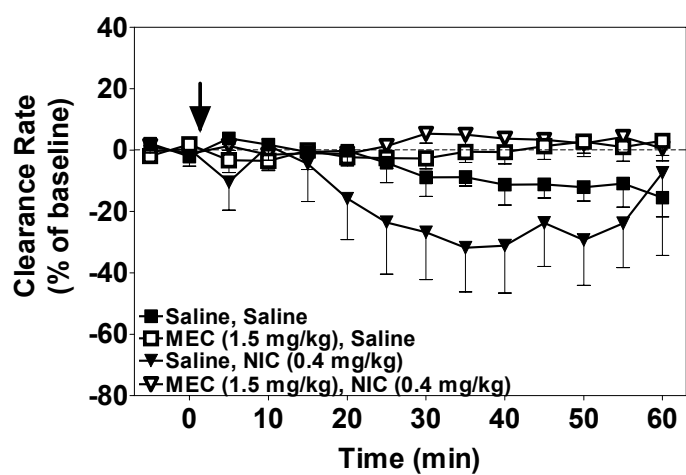
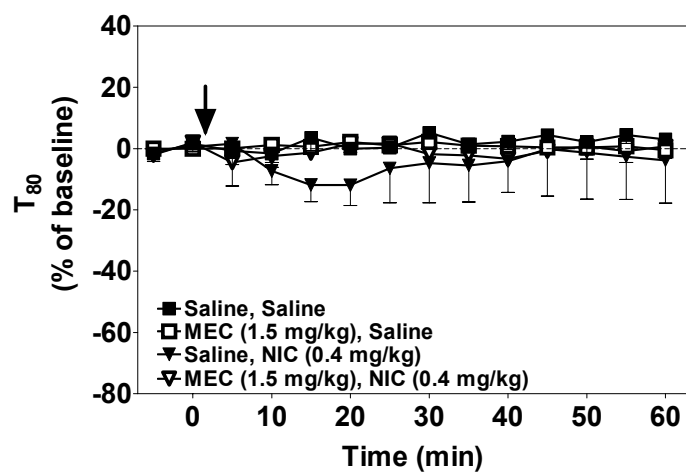
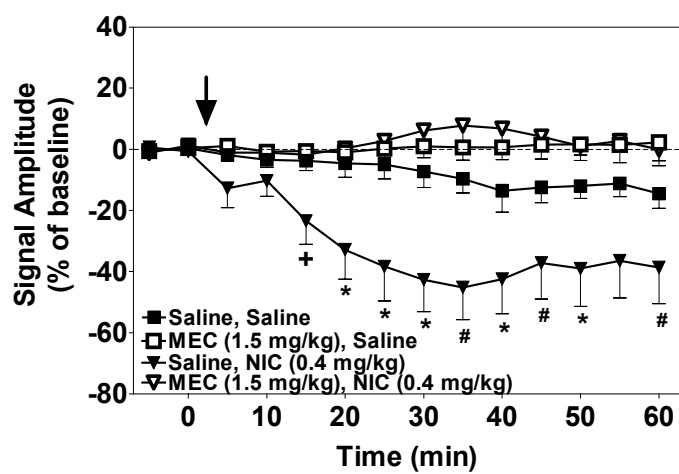


Figure 13. Effect of mecamlamine to inhibit the nicotine-induced decrease in signal amplitude in MPFC. Signal amplitude is shown in the top panel; T_{80} and clearance rate are shown in middle and bottom panels, respectively, for comparison. No significant differences in were observed in T_{80} or clearance rate. After stable baseline signals were obtained in response to pressure ejection of DA (200 μ M) at 5-min intervals, either mecamlamine (MEC; 1.5 mg/kg) or saline was injected s.c. and 40 min later injected s.c. with nicotine (NIC; 0.4 mg/kg) or saline (second injection indicated by the arrow). DA was pressure ejected at 5-min intervals for 60 min following the second injection. Data are expressed as mean, S.E.M. as a percentage of the respective baseline values as a function of time (min). Baseline values for each parameter are indicated in the Results section. Legend indicates treatment group designating first and second injections. (n = 5 - 7 rats/group) * difference between Mecamlamine/Nicotine group and Saline/Nicotine group at the corresponding time point ($p < 0.05$); # difference between Mecamlamine/Nicotine group and Saline/Nicotine group at the corresponding time point ($p < 0.05$; one-tailed); + first time point at which nicotine decreased signal amplitude compared to the corresponding basal.



Chapter Three

Nornicotine Decreases Dopamine Transporter Function In Vivo in Striatum via a Nicotinic Receptor-Mediated Mechanism

I. Introduction

Tobacco use is the number one preventable cause of death in the US (USDHS, 1998), which has prompted the development of cessation pharmacotherapies. Nicotine is believed to be the alkaloid in tobacco that is primarily responsible for chronic tobacco use and dependence (Clarke, 1987; Pomerleau and Pomerleau, 1992). Activation of nicotinic receptors by nicotine results in an increase in the extracellular DA concentrations in brain, which is generally accepted as mediating at least in part, reward produced by nicotine, which subsequently leads to tobacco dependence (Koob, 1992; Corrigall et al., 1992; Stolerman and Jarvis, 1995). The mesocorticolimbic and nigrostriatal DA systems, including the nucleus accumbens, medial prefrontal cortex and striatum and the associated circuitry, have been implicated in drug-induced reward. The nucleus accumbens shell is believed to encode primary appetitive stimuli associated with unconditioned drug reward (Wise and Bozarth, 1987; Bardo, 1998; Koob, 1999; Kelley and Berridge, 2002), including reward produced by nicotine (Fibiger and Phillips, 1987; Corrigall et al., 1992; Mansvelder and McGehee, 2002; Mathieu-Kia et al., 2002). The medial prefrontal cortex encodes secondary conditioned stimuli associated with environmental cues paired with drug, integrating sensory information and leading to reward expectancy, recognized as important to the addiction process and to relapse to drug use

(Berridge and Robinson, 1998; Shima and Tanji, 1998; Kelley and Berridge, 2002; DiChiara et al., 2004; Rose and Behm, 2004; Brody et al., 2004).

Integration of the motivational information from medial prefrontal cortex occurs at least in part in striatum leading to the initiation and execution of movement in reward expectancy and detection of reward (Martin-Soelch et al., 2001). In smokers, nicotine dose-dependently increases neuronal activity in these brain regions assessed using fMRI and PET (Stein et al., 1998; Barrett et al., 2004).

Current tobacco use cessation pharmacotherapies (i.e., bupropion and nicotine) have demonstrated only limited efficacy, since relapse rates are reported to be high (Hurt et al., 2003; George and O'Malley, 2004; Wileyto et al., 2004), indicating a need for the development of alternative, more efficacious smoking cessation therapies. Bupropion acts as an inhibitor of neurotransmitter transporters resulting in increased extracellular DA concentrations (Richelson and Pfenning, 1984; Nomikos et al., 1989; Ascher et al., 1995; Li et al., 2002; Damaj et al., 2004). Recently, bupropion has been reported to also act as a nicotinic receptor antagonist within the same concentration range that it inhibits neurotransmitter transporter function (Fryer and Lukas, 1999; Slemmer et al., 2000; Miller et al., 2002; Gumilar et al., 2003; Damaj et al., 2004). The use of nicotine replacement as a cessation therapy is based on activation of nicotinic receptors resulting in DA release. Nicotine-induced DA release is concentration-dependent and observed in presynaptic terminal regions in both the mesolimbic (Imperato et al., 1986; Rowell et al., 1987; Clarke et al., 1988; Vezina et al., 1992;

Wonnacott, 1997) and nigrostriatal (Westfall et al., 1983; Teng et al., 1997; Wonnacott, 1997; Kaiser et al., 1998; Dwoskin et al., 1999; Vezina et al., 1992) DA systems in both in vivo and in vitro studies. This effect of nicotine is inhibited by mecamylamine, a nonselective and noncompetitive nicotinic receptor antagonist (Mifsud et al., 1989; Nisell et al., 1994a; Sacaan et al., 1995; Teng et al., 1997).

Extracellular DA concentrations are the net result of DA release from the presynaptic terminal and DA uptake back into the terminal by the DA transporter (DAT). Systemic administration of nicotine has been shown to modulate DAT function in vivo in striatum, nucleus accumbens and medial prefrontal cortex, resulting in an increase in DA clearance (Ksir et al., 1995; Hart and Ksir, 1996; Middleton et al., 2004). Nicotine enhancement of DAT function is mediated by nicotinic receptors, as indicated by mecamylamine inhibition of this effect (Middleton, et al., 2004). Considering that DAT function is inhibited by membrane depolarization (Sonders et al., 1997; Huang et al., 1999), it is surprising that nicotine, which depolarizes neurons, enhances DAT function. The nicotine-induced increase in DA release and the concurrent enhancement of DAT function appear to produce a sharpened DA signal. In contrast, when synaptosomes from drug-naïve rats are exposed to nicotine, DAT function is not altered (Zhu et al., 2003), suggesting that nicotine acts at nicotinic receptors located at sites other than on DA terminals to modulate DAT function.

Attention has focused on the *N*-demethylated nicotine metabolite and minor tobacco alkaloid, nornicotine, as contributing to the neuropharmacological effects of nicotine exposure and tobacco use (Crooks and Dwoskin, 1997). Nornicotine inhibits [³H]nicotine binding to rat brain membranes with a 50-fold lower affinity ($K_i = 47$ nM) compared with nicotine ($K_i = 1.0$ nM) (Reavill et al., 1988; Copeland et al., 1991; Zhang and Nordberg, 1993; Xu et al., 2001). In contrast, nicotine and nornicotine exhibit similar affinities for the [³H]methylllycaconitine binding site ($K_i = 770$ nM and 1340 nM, respectively) in brain (Xu et al., 2001). These results indicate interaction of these alkaloids with both $\alpha 4\beta 2^*$ and $\alpha 7^*$ nicotinic receptors. Similar to nicotine, nornicotine evokes a concentration-dependent, Ca^{2+} -dependent and mecamylamine-sensitive increase in DA release from rat striatal and nucleus accumbens slices (Dwoskin et al., 1993; Teng et al., 1997; Green et al., 2001), indicating that nornicotine acts as an agonist at nicotinic receptor subtypes modulating DA release. In the latter studies, whereas nicotine and nornicotine were equipotent in releasing DA from striatal slices, nicotine was 43-fold more potent than nornicotine ($\text{EC}_{50} = 70$ nM and 3.0 μM , respectively) in releasing DA from nucleus accumbens slices. It is interesting to note that nornicotine has a longer half-life than nicotine in plasma and brain (Kyerematen et al., 1990; Crooks et al., 1995, 1997; Ghosheh et al., 2001); and following chronic treatment with nicotine, nornicotine accumulates in brain reaching pharmacologically relevant concentrations (Ghosheh et al., 2001).

In order to more fully elucidate the pharmacological actions of nornicotine, the ability of nornicotine to modulate striatal DAT function was assessed using in vivo voltammetry and in vitro synaptosomal [³H]DA uptake. The hypothesis is that nornicotine, similar to nicotine, will increase DA clearance in the striatum via a nicotinic receptor-mediated mechanism. Thus, the current study provides preclinical data on the pharmacology of nornicotine and provides additional information to assess its potential utility as a new tobacco use cessation agent.

II. Methods

IIa. Chemicals.

[³H]DA (3,4-ethyl-2-[N-³H]-dihydroxyphenylethylamine; specific activity, 27.1 Ci/mmol) was purchased from PerkinElmer Life Science (Boston, MA). S(-)-Nornicotine was prepared as previously described (Swango et al., 1999), and was utilized as the perchlorate salt. S(-)-Nicotine di-d-tartrate, mecamylamine hydrochloride, nomifensine maleate, catechol, pargyline HCl, D-glucose, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) and bovine serum albumin (BSA) were purchased from Sigma/RBI (Natick, MA). Sodium chloride, potassium phosphate, potassium chloride, magnesium sulfate, calcium chloride, and disodium ethylenediamine tetraacetate used in the preparation of the uptake assay buffer were purchased from Fisher Scientific International Inc. (Pittsburgh, PA).

IIb. Subjects

Male Sprague Dawley rats (200–250 g) were obtained from Harlan Laboratories (Indianapolis, IN) and were housed two per cage with free access to food and water in the Division of Lab Animal Resources at the College of Pharmacy, University of Kentucky. Experimental protocols involving the animals were in strict accord with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

IIc. DAT Function In Vivo.

Electrochemical electrodes were prepared and experiments conducted using previously described methods (Middleton et al., 2004). Electrodes contained a single carbon fiber (33 μm diameter) in a pulled glass capillary (4 mm outer diameter, 0.80 mm wall diameter). The exposed carbon fiber extended 50-150 μm beyond the tip of the glass capillary. Electrodes for some experiments were purchased from Quanteon (Lexington, KY). To enhance selectivity for DA, the carbon fiber electrode was coated with Nafion polymer (5% solution, 6-8 coats). Electrodes were calibrated in vitro to determine the sensitivity and selectivity for DA. Calibration curves were generated using a range of DA concentrations (1.0 – 10 μM , at 22 °C in 0.1 M phosphate buffered saline solution, pH 4.0). Electrodes were sensitive to DA and the mean selectivity ratio of DA/ascorbic acid was 606 ± 71 to 1 ($n = 57$). Subsequently, each carbon fiber electrode was attached to a single barrel micropipette (tip diameter of 10-15 μm) with dental wax. The tips of the electrode and

micropipette were positioned 250 - 300 μm apart. Micropipettes were prepared from monofilament glass (1.0 mm O.D., 0.58 mm I.D.) using a vertical pipette puller (Model 720, David Kopf Instruments, Tujunga, CA). Single barreled micropipettes were filled with DA (200 μM , in 154 mM NaCl and 100 μM ascorbic acid, pH 7.4) immediately before conducting the experiment. The concentration of DA for ejection was chosen based on the linear kinetics of DA uptake at this concentration, i.e., DA concentrations that do not saturate DAT in striatum (Zahniser et al., 1999).

Rats were anesthetized with urethane (1.25 - 1.5 g/kg, i.p.) and placed into a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). Body temperature was maintained at 37 °C with a heating pad coupled to a rectal thermometer (Harvard Apparatus, Holliston, MA). The scalp was reflected, and a section of the skull and dura overlying the frontal cortex was removed. A small hole was drilled in the skull above the posterior cortex for placement of two Ag/AgCl reference electrodes, which were cemented into place with dental acrylic. The electrode/micropipette assembly was lowered into the dorsal striatum (1.5 mm anterior to bregma, 2.3 mm lateral from midline, and 4.0 - 5.5 mm below the surface of the cortex) according to the rat brain atlas of Paxinos and Watson (1986). Exogenous DA was pressure ejected (30 - 50 psi, 0.05 ms – 2.5 s) at 5-min intervals using a Picosprizter II (General Valve Corporation, Fairfield, NJ), until reproducible baseline signals (variation in signal amplitude $< \pm 10\%$) were obtained. Using a stereomicroscope fitted with a reticule in one eyepiece,

ejection volume (250 nl/mm) was monitored by measurement of the fluid displaced from the micropipette. High-speed chronoamperometric electrochemical measurements were made continuously (5 times/s, 5 Hz and averaged to 1 Hz), using an electrochemical recording system (IVEC 10; Medical Systems Corporation, Greenvale, NY). The oxidation potential was a square wave of +0.55 volts applied for 100 ms (versus the Ag/AgCl reference electrode), and the resting potential was 0.0 volts for 100 ms. Oxidation currents were digitally integrated during the last 80 ms of each 100-ms pulse.

Dose-response curves for nornicotine modulation of DAT function in striatum were generated, and each animal received only one dose of nornicotine (0, 0.35 – 12.0 mg/kg, s.c.). Data for the nornicotine dose-response were collected as part of a larger study in which the dose-response for nicotine was also determined (Middleton et al., 2004). As such, the saline condition was used as the control for both nicotine and nornicotine dose-response analyses. Nornicotine or vehicle (0.9% saline) was administered once reproducible baseline signals were obtained. Pressure ejection of DA continued at 5-min intervals for 60 min following nornicotine or saline injection.

To determine if nicotinic receptors mediate the response to nornicotine on DA clearance in striatum, the effect of mecamylamine was determined in separate experiments. Once reproducible baseline signals were obtained, rats were injected s.c. with either mecamylamine (1.5 mg/kg) or saline, followed 40

min later by a second s.c. injection of nornicotine (8.0 mg/kg) or saline.

Nornicotine dose was chosen based on the dose-response curves generated in the above experiments, i.e., maximal inhibition of DAT function was observed at 8.0 mg/kg. The mecamylamine dose was chosen based on its ability to inhibit the acute locomotor stimulant effects of nicotine (Miller et al., 2001). DA was pressure ejected every 5 min during the 40 min period following the first s.c. injection and for 60 min following the second s.c. injection. Doses of nornicotine represent the perchlorate salt weight, and the injection volume was 1 ml/kg body weight.

IId. [³H]DA Uptake Assay.

[³H]DA uptake assays were performed using rat striatal synaptosomes as previously described (Teng et al., 1997). Striata from individual rats were homogenized in 20 ml of ice-cold sucrose solution (0.32 M sucrose and 5 mM NaHCO₃, pH 7.4) with 14 passes of a Teflon pestle homogenizer (clearance 0.003 inches). Homogenates were centrifuged (2000g, 10 min, 4°C), and resulting supernatants were centrifuged (20,000g, 15 min, 4°C). Pellets were resuspended in 2.4 ml of ice-cold assay buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgSO₄, 1.25 mM CaCl₂, 1.5 mM KH₂PO₄, 10 mM α-D-glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline, and 0.1 mM ascorbic acid, saturated with 95% O₂/5% CO₂, pH 7.4). Final protein concentration was 20 µg/ml. Assays were performed in duplicate in a total volume of 500 µl. Aliquot parts of synaptosomal suspension (50 µl) were added to tubes containing 350 µl of buffer and 50 µl of

one of nine concentrations of nornicotine or nicotine followed by 50 μ l of [3 H]DA (final concentration, 0.1 μ M). Accumulation proceeded for 10 min at 34°C, and was terminated by addition of 3 ml of ice-cold buffer containing catechol (1 mM), followed by rapid filtration through Whatman GF/B glass fiber filters presoaked with catechol (1 mM). Filters were washed three times with ice-cold buffer, transferred to scintillation vials and radioactivity determined.

IIc. Data Analysis

In vivo voltammetry data are represented as mean values \pm SEM, and *n* represents the number of animals in a treatment group. Three parameters were determined from the DA oxidation currents; A_{\max} , which is defined by the maximal change in extracellular DA concentration; T_{80} , the time for the signal to decay by 80% minus the rise time of the signal; and DA clearance rate (CL_{DA}), which provides a measure of the efficiency of DAT to remove DA from the extracellular space and is defined as the amount of DA pressure ejected, divided by the area under the curve for the DA signal (AUC_{DA}). AUC_{DA} was determined by fitting the decay segment of each DA signal trace following the maximal DA signal amplitude to the following equation: $AUC_{DA} = \sum (y_i + y_{i+1} / 2) * (t_{i+1} - t_i)$; where *y* is the amplitude of the DA signal (micromolar concentration) at any time (*t*, seconds) following DA ejection, and *i* is initial amplitude of the DA signal trace at time zero. AUC_{DA} of the decay segments of DA signals in striatum were determined for the duration of the signal. Thus, CL_{DA} (L/sec) for each DA signal was derived from the amount of ejected DA (moles) divided by the AUC_{DA}

(moles/L/sec) for each DA signal. Figure 14 shows a representative baseline signal from striatum and illustrates the parameters utilized to assess DA clearance. A_{\max} is influenced by the clearance at the point in time at peak DA concentration. CL_{DA} is a time-averaged estimate over the entire interval of the DA signal. Thus, the observed changes in A_{\max} and CL_{DA} following nornicotine injection may not be equivalent.

Nornicotine dose-response curve and time course were analyzed by two-way mixed-factor ANOVA, with dose as a between-groups factor and time as within-subjects factor. A separate ANOVA was performed to analyze the time course for each of the three parameters, A_{\max} , T_{80} and CL_{DA} . To determine the dose-related effect of nornicotine, one-way ANOVAs were conducted on the A_{\max} and CL_{DA} data at each 5-min time point during the 10-60 min period following nornicotine injection. Dunnett's post hoc analysis was performed to determine differences between treatment means and control mean. Additionally, apriori specific within-subject contrasts determined the time point at which nornicotine increased A_{\max} and decreased CL_{DA} compared to baseline (i.e., prior to drug injection).

Analysis of mecamylamine-induced inhibition of the effect of nornicotine on A_{\max} , T_{80} and CL_{DA} was accomplished using separate three-way mixed-factor ANOVAs, with mecamylamine and nornicotine as between-group factors and time as a within-subjects factor. One-way ANOVAs were utilized to assess the

effect of nornicotine and mecamylamine at individual time points. Additionally, apriori specific within-subjects contrasts were performed for each group to determine the time point at which A_{\max} and CL_{DA} increased compared to baseline. ANOVA, specific contrasts and post hoc analyses were performed using SPSS (standard version 11.0, Chicago, IL); $p < 0.05$ two-tailed was considered significant, unless otherwise indicated.

For [3H]DA uptake assays, to generate IC_{50} values for each concentration-response curve nonlinear regression with sigmoidal curve fit were used (GraphPad Prism, version 3.0; GraphPad Software, San Diego, CA).

III. Results

IIIa. Effect of nornicotine on DAT function.

In the previous chapter data were calculated according to methods reported in the literature (Chapter One: Figure 8). To compare the effect of nornicotine on CL_{DA} to the effect of nicotine on CL_{DA} , data from Figure 8 (bottom panel) were re-analyzed using the classical pharmacokinetic measure of DA clearance described in the Methods section. Following systemic administration, nicotine increases CL_{DA} in striatum (Figure 15). Nicotine in a dose-related manner increased CL_{DA} across 55-min recording sessions. Two way repeated measures ANOVA revealed a significant dose x time interaction ($F_{24,156} = 4.87$, $p < 0.001$). Dunnett's post hoc analysis determined that the nicotine (0.8 mg/kg) group was significantly ($p < 0.02$) different from the saline control group. After

stable baseline signals were obtained in response to pressure ejection of DA (200 μ M) at 5-min intervals, either a dose of nicotine (0.6 or 0.8 mg/kg) or saline was injected s.c., and pressure ejection of DA at 5-min intervals continued for 60 min. Compared to the previous method of calculating DA clearance in Figure 8 (Chapter One), the graphical presentation shows the curve projected upward reflecting an increase in DA clearance (increased CL_{DA}).

The effect of systemic administration of nornicotine (0.35 – 12.0 mg/kg) on exogenous DA clearance was determined in the medial dorsal striatum of urethane-anesthetized rats using in vivo voltammetry. Pressure ejection of 200 μ M DA into the striatum at 5-min intervals resulted in stable baseline signals, with an A_{max} of 3.90 μ M (\pm 0.24 μ M; mean \pm SEM), T_{80} value of 29.9 s (\pm 4.09 s) and CL_{DA} of 3.54×10^{-8} L/sec (\pm 0.61×10^{-8} L/sec; n = 32 independent experiments). Once the baseline signal stabilized, rats were injected with either a dose of nornicotine or saline (control); pressure ejection of DA was continued at 5-min intervals for 60 min. The effect of nornicotine on A_{max} , T_{80} and CL_{DA} was determined and compared to that obtained after saline injection. Rise time, duration and decay of the baseline DA signal were not different between nornicotine- and saline-treated rats.

The dose response for nornicotine-induced modulation of DAT function in striatum was determined and the results are shown in Figure 16. A_{max} , T_{80} and CL_{DA} were analyzed using separate two-way ANOVAs. With respect to A_{max} and

CL_{DA}, significant interactions of dose and time were found ($F_{52,351} = 3.58$, $p < 0.001$ and $F_{52,364} = 1.41$, $p < 0.05$; respectively). No significant main effects or interactions were observed for T_{80} . To further analyze the interaction for A_{\max} , separate one-way ANOVAs were conducted on the data at each 5-min time point beginning 10 min after injection of nornicotine or saline (Figure 16, top panel). A nornicotine dose-related increase in A_{\max} was observed; such that significant effects of the 8 mg/kg dose of nornicotine were found at 15-40 min and of the 12 mg/kg dose at 10-60 min ($ps < 0.05$). Within subject comparisons revealed that the A_{\max} for the saline control group gradually decreased by ~15% of baseline during the 55-min period after injection; however, at the 60-min time point, the decrease (~23%) in A_{\max} reached significance. To determine the onset of the effect of nornicotine, specific within-subjects contrasts were performed comparing A_{\max} at each time point following nornicotine injection to A_{\max} at baseline. A significant increase in A_{\max} was observed 20 min following both the 8.0 and 12.0 mg/kg doses of nornicotine ($p < 0.05$). A_{\max} was increased by a maximum of 43% of baseline after the highest dose of nornicotine at the 60 min time point.

One-way ANOVAs were conducted also on the CL_{DA} data to assess the dose-response at each 5-min time point after nornicotine or saline injection (Figure 16, bottom panel). For the group administered saline, CL_{DA} gradually increased by ~25% of baseline across the time course of the session, demonstrating that DA application at 5-min intervals across the 60-min session

resulted in a small increase in DAT function in the control condition. Nornicotine dose-related decreases in CL_{DA} were observed at 15, 25-45 and 55-60 min ($p < 0.05$). Dunnett's post hoc analysis revealed that CL_{DA} was decreased from 25-45 min in the 8 mg/kg group and at 15, 25-45 and 55-60 min in the 12 mg/kg group compared to the control group. Onset of the effect of nornicotine after the 8.0 and 12.0 mg/kg doses on CL_{DA} occurred at 20 and 25 min respectively, following administration. A maximum decrease of 30% was observed after 12 mg/kg, when compared to the within-subject baseline response. Comparing the nornicotine dose-response curves for A_{max} and CL_{DA} reveals that the percent increase in A_{max} was similar to the percent decrease in CL_{DA} , suggesting similar kinetics over the time course of ~ 60 sec (Figure 16).

To ascertain if the effect of nornicotine on DAT function was mediated by nicotinic receptors, the ability of mecamylamine to inhibit nornicotine-induced modulation of A_{max} , T_{80} and CL_{DA} was determined. Pressure ejection of DA (200 μ M) resulted in an A_{max} of 5.78 μ M ($\pm 0.58 \mu$ M; mean \pm SEM), T_{80} value of 17.4 s (± 3.49 s), and CL_{DA} value of 4.82×10^{-8} L/sec ($\pm 0.80 \times 10^{-8}$ L/sec; $n = 25$ independent experiments). Once the baseline signals stabilized, groups of rats were injected with mecamylamine (1.5 mg/kg, s.c.) or saline, and 40 min later with nornicotine (8 mg/kg, s.c.) or saline. DA pressure ejection continued at 5-min intervals for 40 min after the first injection and at 5-min intervals for 60 min after the second injection. Data for the three parameters of DA clearance for these experiments are illustrated in Figure 17.

Three-way ANOVAs on A_{\max} , T_{80} and CL_{DA} data were performed to determine if mecamylamine inhibited the effect of nornicotine on DAT function. A significant three-way interaction of mecamylamine x nornicotine x time was found ($F_{13,325} = 4.04$, $p < 0.001$ and $F_{13,325} = 2.47$, $p < 0.01$) for A_{\max} and CL_{DA} , respectively. No significant interactions or main effects were observed for T_{80} . In the control group injected only with saline, A_{\max} tended to decrease across the 60-min session when compared to its baseline; and significance was reached at 45 min following the second saline injection. For the group administered mecamylamine and saline, no differences were observed across the time course of the session when compared to within-subject baseline response, indicating that mecamylamine alone had no effect on A_{\max} . For the group administered saline and nornicotine, the onset of the nornicotine-induced increase in A_{\max} occurred 25 min after the nornicotine injection (within-subject comparison, $p < 0.05$). At the 60-min time point, nornicotine maximally increased A_{\max} by 32% compared to baseline. To determine if mecamylamine inhibited the effect of nornicotine, one-way ANOVAs were performed to compare the treatment groups at each time point, beginning at the onset of the nornicotine effect (i.e., from 25-60 min). From 45-60 min, A_{\max} for the group administered mecamylamine and nornicotine was significantly less than that for the group administered saline and nornicotine ($p < 0.05$), and not different from the group administered only saline, indicating that mecamylamine completely inhibited the effect of nornicotine.

Similar results were obtained when the CL_{DA} parameter was analyzed (Figure 17, bottom panel). As in the previous experiments described above, one-way ANOVA comparing the groups at each time point revealed that CL_{DA} for the group administered saline and nornicotine was lower at 15-25, 35, 45, and 55 min than that for the control group administered only saline ($p < 0.05$, one tailed). Moreover, CL_{DA} for the group administered saline and nornicotine was lower than the group administered mecamylamine and nornicotine across the same time period. Importantly, the group administered mecamylamine and nornicotine was not different from the control group only administered saline, indicating that mecamylamine completely inhibited the effect of nornicotine.

Comparing the percent change in A_{max} and CL_{DA} for the group administered mecamylamine and saline and for the group administered saline and nornicotine revealed similar percentage changes. However, comparison of the percentage change in A_{max} and CL_{DA} for the saline control group and for the group administered mecamylamine and nornicotine revealed a decrease in A_{max} of 20% for each group, whereas CL_{DA} increased 50% and 60%, respectively. These results appear to be due to greater variability in the CL_{DA} estimates relative to the estimate for A_{max} .

To determine if nicotine and nornicotine were acting at nicotinic receptors located on DA nerve terminals to inhibit DAT function, the [3H]DA uptake assay

was performed using striatal synaptosomes from drug-naïve rats. [³H]DA uptake was not inhibited by either nicotine or nornicotine (IC₅₀ > 100 µM; Table 1).

IV. DISCUSSION

Results from the current *in vivo* voltammetry study show that systemic administration of nornicotine decreased DA clearance in striatum in a dose-related manner, demonstrating that nornicotine inhibits striatal DAT function. The nornicotine-induced decrease in DA clearance was inhibited completely by mecamylamine, a noncompetitive and nonselective nicotinic receptor antagonist, indicating that this effect of nornicotine on DAT function is mediated by nicotinic receptors. In contrast, nornicotine did not inhibit [³H]DA uptake into striatal synaptosomes obtained from drug-naïve rats. Taken together, these results suggest that nornicotine activates nicotinic receptors which are located at sites other than on DA nerve terminals in striatum to inhibit DAT function *in vivo*.

In the current studies mecamylamine was chosen as the nicotinic receptor antagonist to show nicotinic receptor mediation of the effect of nornicotine. The hallmark of an action at nicotinic receptors is blockade by mecamylamine. This is the first approach that the majority of studies use to show nicotinic receptor involvement (Meyer et al., 1997; Papke et al., 2001; Middleton et al., 2004; Damaj et al., 2005; Levin et al., 2005; Sacco et al., 2005). It will be interesting in future studies to examine the effects of subtype selective antagonists on the nornicotine-induced decrease in DA clearance.

Previous studies have shown that nicotine and nornicotine evoke DA release from rat striatal slices (Dwoskin et al., 1993; Teng et al., 1997; Puttfarcken et al., 2000; Wonnacott et al., 2000; Green et al., 2001). Furthermore, in a dose-related manner, nicotine enhances DAT function in striatum, as demonstrated by increased DA clearance using in vivo voltammetry (Middleton et al., 2004). In contrast to nicotine, the current study shows that nornicotine decreases DA clearance in vivo using the same methodological preparation. The observation that nornicotine exhibits antipodal effects compared to nicotine with respect to DAT function in vivo was unexpected and does not support the hypothesis. Potential mechanisms underlying the diametric effects of nicotine and nornicotine on DAT function may be due to the involvement of different nicotinic receptor subtypes modulating DAT function and/or may be associated with differential neural circuitry (see Chapter Six for further discussion).

The current study also shows that both nornicotine and nicotine have no effect on [³H]DA uptake into striatal synaptosomes, which is consistent with previous reports determining the effect of nicotine in vitro (Carr et al., 1989; Zhu et al., 2003). Taken together with the results from the in vivo voltammetry studies, these findings suggest that the nicotinic receptors mediating DAT function in vivo are located on dopaminergic cell bodies in the substantia nigra or in brain regions other than striatum. However, procedural differences between the in vivo and in vitro preparations, such as the use of the urethane anesthetic in vivo, may also contribute to the differential results obtained. Evidence supporting

a critical role for nicotinic receptors at the level of the dopaminergic cell body is provided from studies in which local administration of nicotine into the substantia nigra or ventral tegmental area evokes DA release in striatum and nucleus accumbens, respectively (Blaha and Winn, 1993; Nisell et al., 1994b; Panagis et al., 1996; Sziraki et al., 2002). Additionally, local administration of mecamylamine into the ventral tegmental area was shown to inhibit DA release in the nucleus accumbens following peripheral administration of nicotine (Sziraki et al., 2002). Thus, as has been suggested for nicotine, nicotinic receptors at the level of the substantia nigra may be involved in modulating DAT function in striatum following peripheral nornicotine administration.

DA cell bodies in substantia nigra have mRNAs that express multiple nicotinic receptor subunits ($\alpha 2$ - $\alpha 7$ and $\beta 2$ - $\beta 4$; Klink et al., 2001; Azam et al., 2002; Zoli et al., 2002; Wooltorton et al., 2003). The specific nicotinic receptor subtypes expressed by nigrostriatal neurons are defined by specific combinations of subunits expressed. Pair-wise expression of nicotinic receptor subunits in *Xenopus* oocytes initially revealed characteristic pharmacological profiles, i.e., relative sensitivity and responsiveness to a range of nicotinic agonists and antagonists (Luetje and Patrick, 1991). Furthermore, inclusion of a third type of subunit (e.g., $\alpha 5$ with $\alpha 3$ and $\beta 2$) in cell expression systems resulted in an altered responsiveness (i.e., calcium permeability and desensitization characteristics) when compared with pair-wise subunit expression (Gerzanich et al., 1998). Moreover, patch-clamp results from neurons in midbrain slices revealed four

different patterns of nicotinic receptor-mediated currents, suggesting stimulation of structurally complex native nicotinic receptors potentially containing combinations of as many as four different subunits (Klink et al, 2001). Furthermore, nicotinic receptor subtypes expressed at the level of the substantia nigra may be different from those at the terminals in striatum (Klink et al., 2001; Azam et al., 2002; Zoli et al., 2002; Woollorton et al., 2003), which also could explain the lack of nornicotine inhibition of DA uptake using striatal synaptosomes. More than one nicotinic receptor subtype has been suggested to mediate nicotine-evoked DA release in striatum (Kulak et al., 1997; Kaiser et al., 1998; Dwoskin et al., 2004). The current evidence provides the first report that more than one nicotinic receptor subtype may mediate DAT function, since nicotine and nornicotine both mediate DAT function but in a qualitatively different manner.

Nicotine is an effective pharmacotherapy for the cessation of tobacco smoking when used alone or in combination with other drugs or clinical programs (Po, 1993; Rose et al., 1994; Balfour and Fagerstrom, 1996; Glover and Glover, 2001; Karnath, 2002). However, despite efficacy with nicotine replacement, the majority of smokers continue to relapse, suggesting that more efficacious therapeutic agents are needed. Nornicotine may offer a beneficial alternative to nicotine as a tobacco use cessation agent. The pharmacokinetic profile of nornicotine, with its longer half-life and slower clearance compared to nicotine (Kyerematen et al., 1990; Crooks and Dwoskin, 1997; Ghosheh et al., 1999), may

afford additional advantages over nicotine. Furthermore, since nornicotine is significantly less potent than nicotine in increasing blood pressure and heart rate (Mattila, 1963; Risner et al., 1988; Stairs et al., submitted 2005), the safety index for nornicotine may be greater than that for nicotine, especially among smokers with advanced cardiovascular disease. Moreover, the ability of nornicotine to decrease DAT function in vivo, as shown in the present study, may afford another advantage over nicotine as a tobacco use cessation agent. In this respect, the antidepressant agent, bupropion, which inhibits both DAT and norepinephrine transporter function (Ascher et al., 1995), has been shown to be efficacious as a tobacco smoking cessation agent (Hurt et al., 1997; Jorenby et al., 1999; Shiffman et al., 2000). Thus, nornicotine incorporates both inhibition of DAT function and promotion of DA release properties into one molecule, and these pharmacological effects have been associated with the clinical efficacy of the currently available tobacco cessation products, nicotine and bupropion.

Behavioral studies using animal models also provide support for the use of nornicotine as a tobacco use cessation agent. Nornicotine produces nicotine-like discriminative stimulus effects (Rosecrans and Meltzer, 1981; Goldberg, et al., 1989; Bardo et al., 1997; Desai et al., 1999), as well as nicotine-like effects on schedule-controlled operant responding (Risner et al., 1985; 1988). Recent results indicate that nornicotine functions as a positive reinforcer (Bardo et al., 1999); however, under similar experimental conditions, nornicotine is associated with a lower rate of responding in comparison with nicotine (Corrigall and Coen,

1989; Donny et al., 1995; Bardo et al., 1999), suggesting that nornicotine has a lower reinforcing efficacy. Moreover, nornicotine has been shown to decrease self-administration of nicotine in rats (Green et al., 2000). Furthermore, across repeated nornicotine pretreatments, tolerance did not develop to nornicotine-induced decrease in nicotine self-administration. Thus, a simple structural change, i.e., removal of the N-methyl group from the pyrrolidine ring nitrogen of nicotine which affords nornicotine, causes a profound change in its effect on DAT function, which may be beneficial with respect to its pharmacological profile. In summary, the current preclinical results suggest that nornicotine could be a promising candidate for development as a smoking cessation agent.

Figure 14. Representative baseline DA signal in striatum of urethane-anesthetized rats prior to nicotine or saline challenge. Electrochemical signals were obtained following pressure ejection of DA (200 μM) and represent the baseline response. Three parameters are obtained from each DA signal; A_{max} , T_{80} and CL_{DA} . Data are expressed as μM DA as a function of time (sec) after DA pressure ejection. DA concentrations were calculated based on calibration curves generated in vitro.

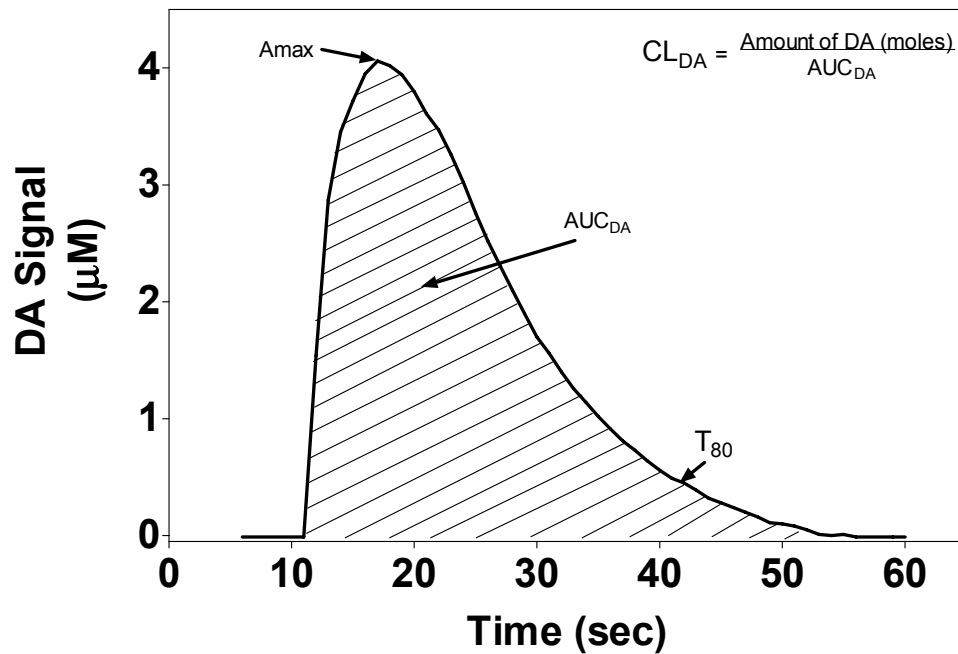


Figure 15. Following systemic administration, nicotine increases CL_{DA} in striatum. Nicotine in a dose-related manner increased CL_{DA} across 55-min recording sessions. After stable baseline signals were obtained in response to pressure ejection of DA (200 μ M) at 5-min intervals, either a dose of nicotine (0.6 or 0.8 mg/kg) or saline was injected s.c. (indicated by the arrow), and pressure ejection of DA at 5-min intervals continued for 60 min. Data are expressed as mean, S.E.M. as a percentage of the respective baseline values as a function of time (min). (n = 4 - 7 rats/group) *indicates significant difference from control.

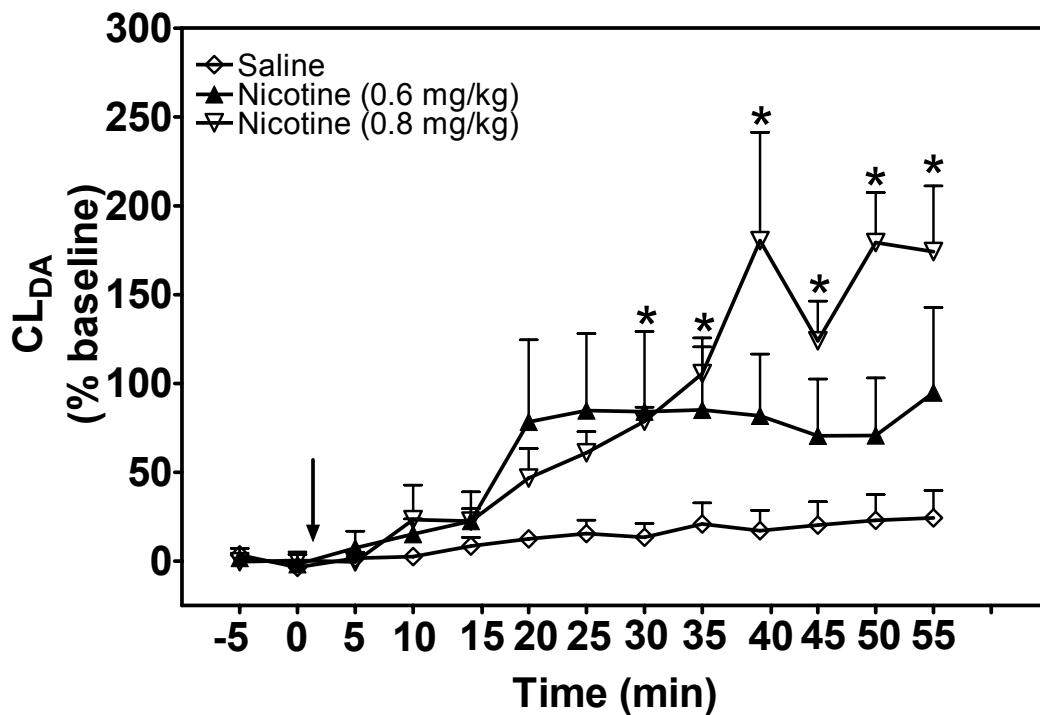


Figure 16. Dose-response for systemically administered nornicotine to alter DA signal amplitude (top panel), T_{80} (middle panel), and CL_{DA} (bottom panel) in striatum of urethane-anesthetized rats. Nornicotine in a dose-related manner increased signal amplitude and decreased CL_{DA} , but did not significantly alter T_{80} across 60-min recording sessions. After stable baseline signals were obtained in response to pressure ejection of DA (200 μ M) at 5-min intervals, either a dose of nornicotine (0.35 – 12.0 mg/kg) or saline was injected s.c. (indicated by the arrow), and pressure ejection of DA at 5-min intervals continued for 60 min. Data are expressed as mean \pm SEM as a percentage of the respective baseline values as a function of time (min). Mean baseline values for each parameter are provided in the Results section. *indicates significant difference between the norNIC (8.0 mg/kg) group and saline; # indicates significant difference between the norNIC (12.0 mg/kg) group and saline; norNIC, nornicotine; n = 6 - 7 rats/group.

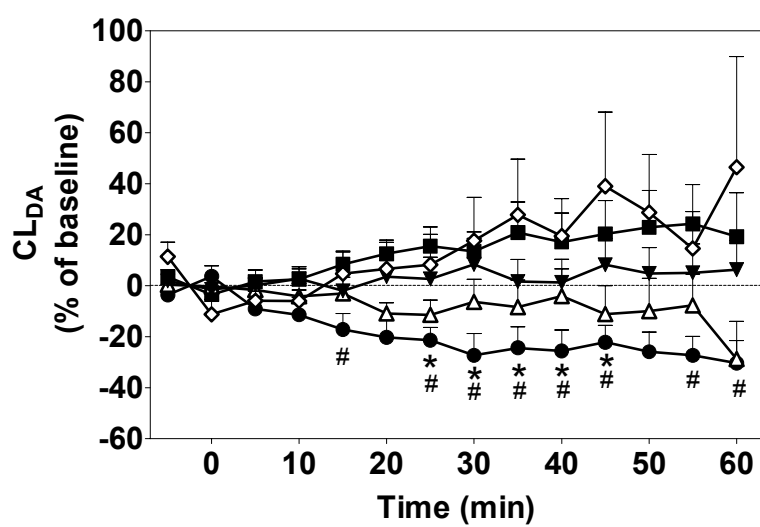
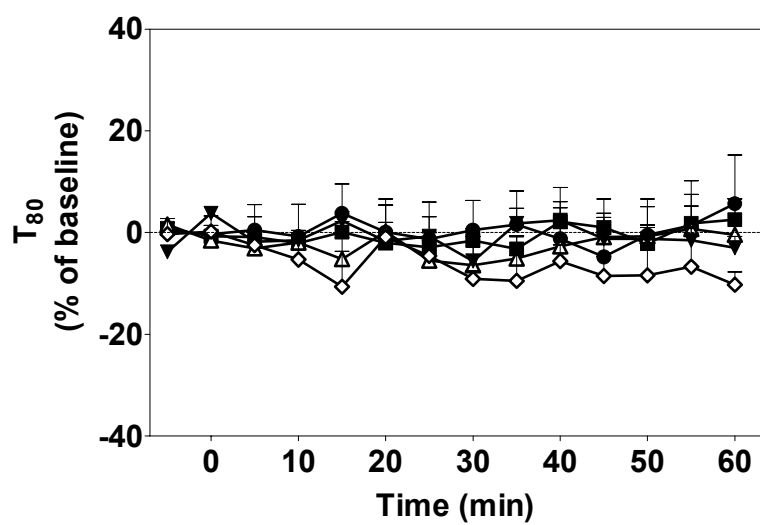
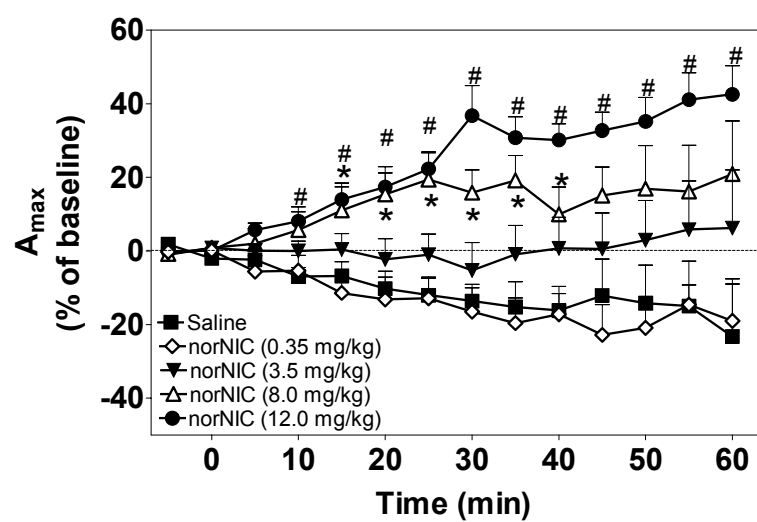


Figure 17. Effect of mecamlamine to inhibit the nornicotine-induced increase in signal amplitude in striatum. Signal amplitude is shown in the top panel; T_{80} and CL_{DA} are shown in middle and bottom panels, respectively, for comparison. After stable baseline signals were obtained in response to pressure ejection of DA (200 μ M) at 5-min intervals, either mecamlamine (1.5 mg/kg) or saline was injected s.c., and 40 min later nornicotine (8.0 mg/kg) or saline was injected s.c.(second injection indicated by the arrow). DA continued to be pressure ejected at 5-min intervals for 60 min following the second injection. Data are expressed as mean \pm SEM as a percentage of the respective baseline values as a function of time (min). Baseline values for each parameter are indicated in the Results section. Legend indicates treatment group designated as first/second injection: saline/saline, saline/nornicotine, mecamlamine/saline and mecamlamine/nornicotine groups. norNIC, nornicotine; MEC, mecamlamine; *indicates significant difference between the saline,norNIC group and mec-norNIC group; n = 7 - 9 rats/group.

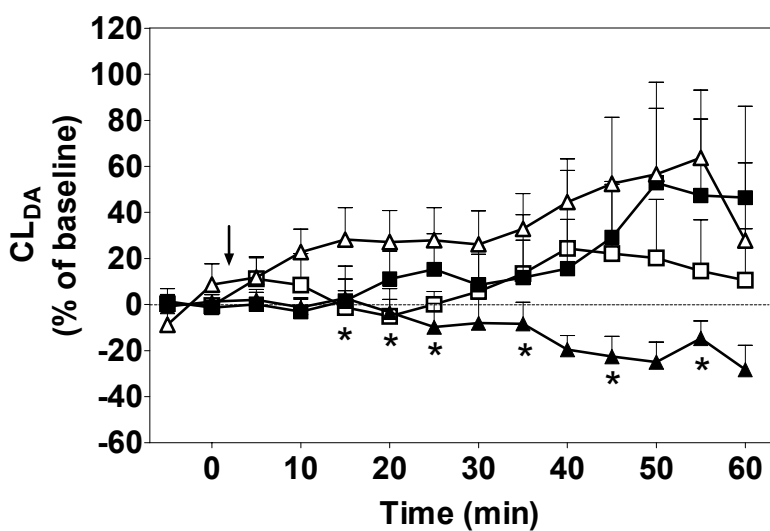
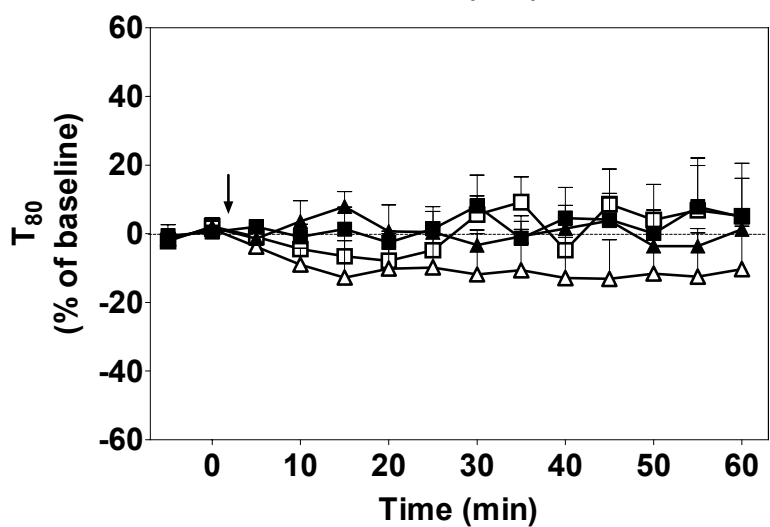
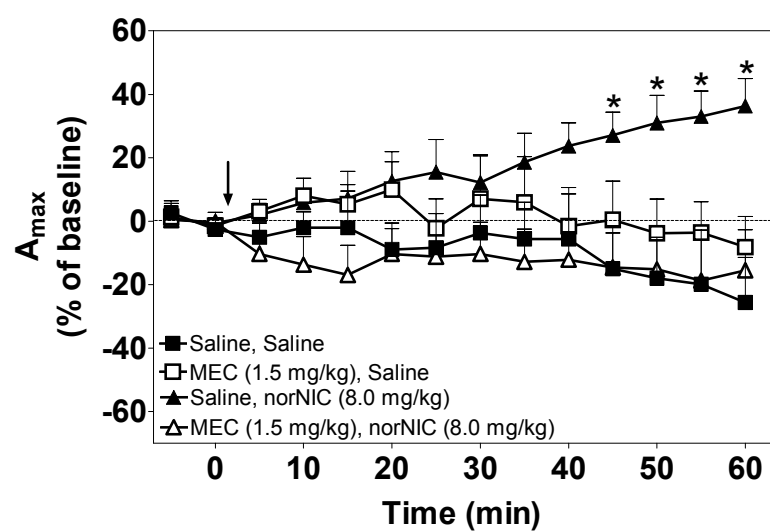


Table 1. Nicotine and nornicotine do not inhibit [³H]DA uptake into striatal synaptosomes.

	[³H]DA Uptake (pmol/min/mg) mean ± S.E.M.						
Drug	Control	1 nM	10 nM	100 nM	1 µM	10 µM	100 µM
Nicotine	18.6±1.9	16.6±1.6	17.4±2.6	18.2±2.4	18.0±1.8	17.6±1.8	15.2±1.3
Nornicotine	10.9±1.6	10.2±1.7	10.9±1.8	10.6±1.4	10.8±1.4	10.4±1.0	10.7±1.1

Chapter Four

Tolerance Develops to the Nicotine-Induced Increase in Dopamine Transporter Function

I. Introduction

Nicotine is generally accepted to be the active alkaloid in tobacco responsible for the reinforcing effects of tobacco (Clarke, 1987; Pomerleau and Pomerleau, 1992). The mesocorticolimbic DA system has been implicated in the rewarding properties of drugs of abuse leading to addiction (Tzschentke, 2001). Dependence liability for nicotine stems from its intrinsic reinforcing properties and the result of activation of DA pathways in brain (Fibiger and Phillips, 1987; Corrigall et al., 1992, 1994; Balfour and Benwell, 1993). The mesocorticolimbic and nigrostriatal DA systems, including the nucleus accumbens, MPFC and striatum and the associated circuitry, have been implicated in drug-induced reward. The nucleus accumbens shell encodes primary appetitive stimuli associated with unconditioned drug reward (Kelley and Berridge, 2002), including that produced by nicotine (Corrigall et al., 1992; Mathieu-Kia et al., 2002). The MPFC encodes secondary conditioned stimuli associated with environmental cues which have been paired with drug, and integration of the motivational information from MPFC occurs at least in part in striatum leading to the initiation and execution of movement in expectancy and detection of reward (Kelley and Berridge, 2002; Martin-Soelch et al., 2001).

Nicotine releases DA from its presynaptic terminals in a concentration-dependent manner in both the mesolimbic (Clarke et al., 1988; Vezina et al., 1992; Marshall et al., 1997; Puttfarcken et al., 2000; Grady et al., 2002; Bednar et al., 2004) and nigrostriatal (Giorguieff-Chesselet et al., 1979; Grady et al., 1992, 1994; Harsing et al., 1992; Vezina et al., 1992; El-Bizri & Clarke, 1994; Marshall et al., 1997; Teng et al., 1997; Kaiser et al., 1998; Puttfarcken et al., 2000; Wonnacott et al., 2000; Zhou et al., 2001; Grady et al., 2002; Mogg et al., 2002; Rice and Cragg, 2004) DA systems. Nicotine-evoked DA release is inhibited by mecamylamine, a nicotinic receptor antagonist (Teng et al., 1997). Nicotinic receptors are located on cell bodies and nerve terminals of dopaminergic pathways (Clarke and Pert, 1985; Varanda et al., 1985; Banerjee et al., 1990; Wonnacott, 1997).

Although there are some studies (Grilli et al., 2005) that found that 10 days of nicotine treatment did not change the releasing effect of nicotine in striatal or accumbal synaptosomes, i.e. nicotine released the same amount of dopamine after chronic treatment compared to acute nicotine, the majority of studies show an enhanced DA release following chronic nicotine. In a number of studies, the effect of nicotine to release DA has been shown to be enhanced following repeated nicotine administration compared with its acute administration. Following 10 days of nicotine pretreatment, nicotine-evoked DA release from superfused striatal slices was greater than nicotine-evoked DA release from a control group of rats injected with saline for ten days (Yu and Wecker, 1994). In

in vivo microdialysis studies, nicotine-evoked DA release in the prefrontal cortex and striatum was enhanced in rats pretreated with nicotine for 7 or 12 days, compared nicotine-evoked DA release from rats receiving acute nicotine (Nisell et al., 1996; Marshall et al., 1997). Also in nucleus accumbens, extracellular DA levels in rats pretreated once daily for 5 days with nicotine were increased following nicotine challenge compared to the DA levels in rats acutely administered nicotine (Benwell et al., 1995). A study examining the chronic use of tobacco in humans determined that DA levels were elevated in the striatum of smokers compared to nonsmokers (Court et al., 1998). Although the effects of chronic nicotine on DA release have been studied, the effect of repeated nicotine on DA transporter (DAT) function and the role that DAT plays in enhancing extracellular DA concentrations in response to repeated nicotine have not been examined.

Nicotine-induced alterations in DAT function may contribute to the observed nicotine-induced increase in extracellular DA concentrations described above. DAT functions to regulate extracellular DA concentrations, and DAT is a target for psychostimulant drugs of abuse. For example, amphetamine increases extracellular DA concentrations directly through an interaction with DAT, promoting reverse transport of DA (Liang and Rutledge, 1992; Sulzer et al., 1995). Cocaine inhibits DAT function, also resulting in increased extracellular DA concentrations (Kuhar et al., 1991). Additionally, drugs indirectly alter DAT function through activation of specific receptors, e.g., raclopride inhibits DA D2

receptors which indirectly inhibit DAT function (Cass and Gerhardt, 1994) and NMDA stimulates ionotropic glutamate receptors to increase DAT function (Welch and Justice, 1996). Acutely, nicotine also indirectly augments DAT function through activation of nicotinic receptors (Middleton et al., 2004). In studies using high speed chronoamperometric recordings to assess DA clearance *in vivo*, acute systemic administration of nicotine dose-dependently increased DA clearance in nucleus accumbens, striatum and MPFC of anesthetized rats (Hart and Ksir, 1996; Middleton et al., 2004), and the effect of nicotine in MPFC and striatum was inhibited by mecamylamine, a noncompetitive, nonselective nicotinic receptor antagonist (Middleton et al., 2004).

The goal of the current study was to test the hypothesis that repeated nicotine administration will further enhance the nicotine-induced increase in DA clearance in striatum and MPFC. The results of these studies thereby begin to delineate the role that DAT plays in enhancing extracellular DA concentrations in response to repeated nicotine administration.

II. Methods

Ila. Materials. S(-)-Nicotine ditartrate (nicotine), 3-hydroxytyramine hydrochloride (dopamine, DA), mecamylamine HCl (mecamylamine), desipramine hydrochloride and sodium phosphate dibasic were purchased from Sigma-Aldrich (Natick, MA). Sodium phosphate monobasic, sodium chloride, ascorbic acid and urethane were purchased from Fisher Scientific (Pittsburgh,

PA). Nafion perfluorinated ion-exchange resin was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dental wax was purchased from Patterson Dental Supply, Inc. (Louisville, KY). Dental acrylic was purchased from CMA Microdialysis (Acton, MA). Epoxylite and PX grade Graphpoxy were purchased from Epoxylite Corp. (Irvine, CA) and Dylon Industries, Inc. (Cleveland, OH), respectively. Carbon fibers of 30 μ m diameter were purchased from Textron, Inc. (Lowell, MA), and 28 gauge lacquer-coated copper wire was purchased from Radio Shack (Lexington, KY).

IIb. Animals. Male Sprague Dawley rats (200 – 250 g) were obtained from Harlan Laboratories (Indianapolis, IN) and were housed two per cage with free access to food and water in the Division of Lab Animal Resources at the College of Pharmacy, University of Kentucky. Experimental protocols involving the animals were in strict concordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

IIc. Treatment Protocol. Groups of rats were pretreated (s.c.) with nicotine (0.4 or 0.8 mg/kg, 1 ml/kg, for MPFC and striatal experiments, respectively) or saline once daily for 5 days. Twenty-four hours after the last nicotine or saline injection, *in vivo* voltammetry was performed. During the voltammetry experiment, rats were injected with a challenge dose of either nicotine or saline. For MPFC experiments, rats received a challenge injection of

either nicotine (0.4 mg/kg, salt weight) or saline. For striatal experiments, rats received a challenge injection of either nicotine (0.8 mg/kg, salt weight) or saline. Thus, for each brain region, 4 groups of rats were used and are designated by pretreatment/challenge injections, i.e., saline/saline, saline/nicotine, nicotine/saline, nicotine/nicotine.

IId. *In Vivo* Electrochemical Measurements. Clearance of exogenously applied DA using *in vivo* voltammetric measurements has been previously shown to reliably evaluate DAT function (Wightman et al., 1988; Cass et al., 1992). Rats were anesthetized with urethane (1.25 - 1.5 g/kg, i.p.) and placed into a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). Previous studies from our laboratory have shown that a systemic injection of nicotine (0.4 and 0.8 mg/kg salt weight for MPFC and striatum, respectively) increased DA clearance by ~50% (Middleton et al., 2004). Thus, these doses of nicotine were chosen for the current study to examine the effect of repeated nicotine administration on DA clearance. Body temperature was maintained at 37 °C with a heating pad coupled to a rectal thermometer (Harvard Apparatus, Holliston, MA). The scalp was reflected, and a section of the skull and dura overlying the frontal cortex was removed. A small hole was drilled in the skull above the posterior cortex for placement of two Ag/AgCl reference electrodes, which were cemented into place with dental acrylic.

Electrochemical electrodes were prepared and electrochemical experiments were conducted using previously described methods (Middleton, et al., 2004). Electrochemical recording electrodes contained a single carbon fiber (33 μm diameter) in a pulled glass capillary (4 mm outer diameter, 0.80 mm wall diameter) sealed with Epoxylite. Graphite epoxy resin and a 28 gauge lacquer-coated copper wire were inserted into the glass capillary to establish electrical contact with the carbon fiber. The exposed carbon fiber extended 50-150 μm beyond the tip of the glass capillary. Electrodes for some experiments were purchased from Quanteon (Lexington, KY). To enhance selectivity for DA, the carbon fiber electrode was coated with Nafion polymer (5% solution, 6 - 8 coats) and cured by heating at 250 $^{\circ}\text{C}$ for 5 min. Electrodes were calibrated *in vitro* and calibration curves were generated using a range of DA concentrations (1.0 – 10 μM , at 22 $^{\circ}\text{C}$ in 0.1 M PBS solution, pH 4.0) to determine the sensitivity and selectivity for DA. Electrodes were sensitive to DA and the mean selectivity ratio of DA/ascorbic acid was 3806 ± 549 to 1 ($n = 69$). Each carbon fiber electrode was attached to a single barrel micropipette (tip diameter of 10-15 μm) with dental wax. The electrode and micropipette were positioned 250 - 300 μm apart. Micropipettes were prepared from monofilament glass (1.0 mm O.D., 0.58 mm I.D.) using a vertical pipette puller (Model 720, David Kopf Instruments, Tujunga, CA). Single barreled micropipettes were filled with DA (200 μM , in 154 mM NaCl and 100 μM ascorbic acid, pH 7.4) immediately prior to conducting the experiments. The concentration of DA for ejection was chosen based on the

linear kinetics of DA uptake at this concentration, i.e., DA concentrations that do not saturate DAT in striatum (Zahniser et al., 1999).

The electrode/micropipette assembly was lowered into the MPFC (2.9 mm anterior to bregma, 1.0 mm lateral from midline, and 2.5 – 5.0 mm below the surface of the cortex) or dorsal striatum (1.5 mm anterior to bregma, 2.3 mm lateral from midline, and 4.0 - 5.5 mm below the surface of the cortex) according to the rat brain atlas of Paxinos and Watson (1986). Exogenous DA was pressure ejected at 5-min intervals using a Picosprizter II (General Valve Corporation, Fairfield, NJ). Using a stereomicroscope fitted with a reticule in one eyepiece, ejection volume (250 nl/mm) was monitored by measurement of the fluid displaced from the micropipette. The volume and amount of DA pressure ejected was constant within each experiment, but varied between experiments (12.5 – 200 nl, 2.5 – 40 pmol, 30 - 50 psi, 0.05 ms - 2.5 s). Ejection volume for each experiment was chosen to provide reproducible baseline signals with a maximum peak height of ~4 μ M (variation in signal amplitude $< \pm 10\%$). High-speed chronoamperometric electrochemical measurements were made continuously (5 times/s, 5 Hz and averaged to 1 Hz), using an electrochemical recording system (IVEC 10; Medical Systems Corporation, Greenvale, NY). The oxidation potential was a square wave of +0.55 volts applied for 100 ms (versus the Ag/AgCl reference electrode), and the resting potential was 0.0 volts for 100 ms. Oxidation currents were digitally integrated during the last 80 ms of each 100-ms pulse. Electrodes were stable throughout the time course of the

experiment, maintaining sensitivity and selectivity for DA. In some cases, electrodes were used for multiple experiments.

For each experiment, nicotine or saline challenge injection (s.c.) was administered once reproducible baseline signals were obtained. Pressure ejection of DA continued at 5-min intervals for 60 min following nicotine or saline injection. In experiments performed in MPFC, rats were pretreated with desipramine (25 mg/kg, s.c.) to block DA uptake via the norepinephrine transporter (Carboni et al., 1990).

Ile. Data and Statistical Analysis. *In vivo* voltammetry data are represented as mean values \pm SEM, and n represents the number of animals in a treatment group. Figure 18 shows a representative baseline signal obtained from MPFC and illustrates the parameters utilized to assess DA clearance. Three parameters were determined from the DA oxidation currents; A_{max} , which is defined by the maximal change in extracellular DA concentration; T_{80} , the time for the signal to decay by 80% minus the rise time of the signal; and DA clearance rate (CL_{DA}), which provides a measure of the efficiency of DAT to remove DA from the extracellular space and is defined as the amount of DA pressure ejected, divided by the area under the curve for the DA signal (AUC_{DA}). AUC_{DA} was determined by fitting each DA signal trace following DA application to the following equation: $AUC_{DA} = \sum (y_i + y_{i+1} / 2) * (t_{i+1} - t_i)$; where y is the amplitude of the DA signal (micromolar concentration) at any time (t , seconds) following DA ejection, and where i is an integral number from 1 to n that starts at the initial time point where the amplitude of the DA signal trace moves above the baseline

level. AUC_{DA} of the DA signals were determined for the duration of each DA signal trace. Thus, CL_{DA} (L/s) for each DA signal was derived from the amount of ejected DA (pmoles) divided by the AUC_{DA} (s * pmoles/L) for each DA signal and is based on classical pharmacokinetic analysis (Ito et al., 1998; Shargel et al., 2005). The half-life or its reciprocal (i.e., the rate constant K_T) which has been used to describe DA elimination is dependent upon both the volume into which the DA distributes as well as the clearance of DA. The current study focused on CL_{DA} , which is a physiological measurement of DA elimination independent of the amount or volume of DA that was pressure ejected and independent of the volume into which the DA distributes under linear conditions. Thus, CL_{DA} is a direct determination of the physiological process(es) responsible for the removal of DA from the pressure ejection site.

A_{max} , expressed as amount (μM) at a point in time, is a single measurement that defines when the rate of DA dispersal from the ejection site to the detector electrode becomes exactly balanced by all rate processes (uptake and/or diffusion) that act to lower DA concentrations at a single instant in time. As such, A_{max} represents an instantaneous composite of DA dispersion, diffusion and uptake processes. CL_{DA} is a time-averaged estimate over the entire interval of the DA signal that more effectively defines the average efficiency of DA uptake into neurons. Thus, drug-induced changes in A_{max} and CL_{DA} , which measure different components of DA disposition, need not be equivalent.

Analysis of the effect of repeated nicotine administration on each of the three parameters (A_{\max} , T_{80} and CL_{DA}) for both brain regions was accomplished using separate three-way mixed-factor ANOVAs, with pretreatment and challenge as between-group factors and time as a within-subjects factor. In both MPFC and striatum, significant interactions were found for the data expressed as A_{\max} and CL_{DA} such that one-way ANOVAs were utilized subsequently to assess the effect of repeated nicotine at individual time points. Additionally, specific within-subjects contrasts were performed to determine the time point at which nicotine significantly altered A_{\max} and CL_{DA} compared to baseline. ANOVA, specific contrasts and post hoc analyses were performed using SPSS (standard version 11.0, Chicago, IL). $p < 0.05$ was considered significant, two-tailed. Graphs were generated using GraphPad Prism software (Prism v3.0, GraphPad software, Inc., San Diego, CA).

III. Results

IIla. Effect of Repeated Nicotine Administration on Clearance of Exogenous DA in MPFC. The effect of repeated nicotine (0.4 mg/kg) or saline administration (s.c.) on clearance of exogenous DA was determined in the MPFC using *in vivo* voltammetry. Pressure ejection of 200 μ M DA into the MPFC at 5 min intervals resulted in stable baseline signals, with an A_{\max} value of 4.44 μ M (\pm 0.20 μ M; mean \pm S.E.M), T_{80} value of 64.6 s (\pm 5.61 s), and CL_{DA} of 4.40×10^{-8} L/s (\pm 0.67×10^{-8} L/s; $n = 33$ independent experiments). Once baseline signals stabilized, rats were injected with either a challenge dose of nicotine (0.4 mg/kg)

or saline; DA pressure ejection continued at 5-min intervals for 60 min. The effect of the challenge dose of nicotine on A_{\max} , T_{80} and CL_{DA} was determined and compared to that obtained after saline injection (Figure 19). Rise time, duration and decay of the baseline DA signal were not different between the rats repeatedly treated with nicotine compared to the saline control rats repeatedly injected with saline.

The ability of a challenge dose of nicotine to alter DA clearance in MPFC in rats either repeatedly injected with nicotine or saline was determined and the data for each parameter (A_{\max} , T_{80} and CL_{DA}) analyzed using separate three-way ANOVAs. No significant main effects or interactions were observed for T_{80} . With respect to A_{\max} , the 3-way pretreatment x challenge x time interaction was not significant; however, the pretreatment x time interaction ($F_{13, 377} = 5.35$, $p < 0.001$) and the challenge x time interaction ($F_{13, 377} = 2.41$, $p < 0.01$) were significant. Analysis of the data from the group of rats receiving saline once daily for 5 days and then a saline challenge on the experiment day (saline control group) revealed that compared to baseline, A_{\max} gradually decreased by ~18% during the 60-min period, reaching significance at 25 min and at 35-60 min following the saline challenge injection (Figure 19, top panel). Thus, in the saline control group, DA application at 5-min intervals for 60 min resulted in a small, but significant, decrease in A_{\max} .

Interestingly, this effect only occurred in the saline control group (rats injected with saline for 5 days and challenged with saline on the experiment day). In the groups repeatedly injected with nicotine (both when challenged with saline or nicotine on the experiment day) no significant decreases were observed in A_{\max} . It appears that repeated nicotine treatment produces tolerance to the increase in DAT function that was observed following saline injection.

Between groups comparisons also revealed that A_{\max} for the group of rats injected with saline for 5 days and challenged with nicotine on the experiment day (the nicotine control group) was significantly different ($p < 0.01$) from all of the other treatment groups (Figure 19, top panel). Subsequent one-way ANOVAs conducted at each 5-min time point from 10-60 min following challenge injection revealed that A_{\max} values for the nicotine control group were different ($p < 0.05$) from all other treatment groups during the period from 45-60 min. Compared to the baseline response (within-subject comparisons), the onset of the nicotine-induced decrease in A_{\max} for the nicotine control group was 20 min following nicotine challenge, and a 41% decrease in A_{\max} was observed at the 60 min time point in this group. In contrast, within-subject comparisons also revealed that A_{\max} values did not differ from baseline across the 60 min session for the group injected with nicotine for 5 days followed by the nicotine challenge. Moreover, from 45-60 min, A_{\max} was decreased in the nicotine control group compared to the group repeatedly injected with nicotine and then challenged with nicotine on the experimental day. Furthermore, no differences were observed in

A_{\max} values between the group repeatedly injected with nicotine and challenged with nicotine and the saline control group. Taken together, these results indicate that repeated treatment with nicotine resulted in tolerance to the effect of nicotine to decrease A_{\max} .

Analysis of the effect of the nicotine challenge on CL_{DA} revealed a significant pretreatment x time interaction ($F_{13,377}$, $p < 0.01$) and challenge x time interaction ($F_{13,377}$, $p < 0.05$). With respect to the saline control group, there were no significant changes in CL_{DA} during the 60 min recording period following saline injection compared to baseline, although there was a trend for CL_{DA} to increase (Figure 19, bottom panel). With respect to the nicotine control group, compared to baseline, the onset of the nicotine-induced increase in CL_{DA} occurred at 25 min after nicotine challenge ($p < 0.05$); and nicotine increased CL_{DA} by 59% compared to the saline control group at the 55-min time point. In contrast, within-subject comparisons revealed that CL_{DA} did not differ from baseline across the 60 min session in the group repeatedly treated with nicotine and then challenged with nicotine on the experimental day. Between groups comparisons also revealed that the nicotine control group was significantly different from the group repeatedly injected with nicotine and then challenged with nicotine ($p < 0.01$). Separate one-way ANOVAs conducted at each time point from 10-60 min revealed that at 50 and 55 min, CL_{DA} in the nicotine control group was significantly greater (81%) than that in the group repeatedly injected with nicotine followed by nicotine challenge. Furthermore, no significant

differences were observed in CL_{DA} values between the nicotine challenge group which was repeatedly injected with nicotine and the saline control group. These results indicate that repeated treatment with nicotine resulted in tolerance to the effect of nicotine to increase CL_{DA} in MPFC.

IIIb. Effect of Repeated Nicotine Administration on Clearance of Exogenous DA in Striatum. The effect of repeated nicotine (0.8 mg/kg) or saline administration (s.c.) on clearance of exogenous DA was determined in the striatum. Pressure ejection of 200 μ M DA into striatum at 5 min intervals resulted in stable baseline signals, with an A_{max} value of 4.28 μ M (\pm 0.14 μ M; mean \pm S.E.M.), T_{80} value of 18.8 s (\pm 2.51 s) and CL_{DA} of 0.218 L/s (\pm 0.028 L/s; n = 33 independent experiments). Once baseline signals stabilized, rats were injected with either a challenge dose of nicotine (0.8 mg/kg) or saline; DA pressure ejection continued at 5-min intervals for 60 min. The effect of the challenge dose of nicotine on A_{max} , T_{80} and CL_{DA} was determined and compared to that obtained after saline injection (Figure 20). Rise time, duration and decay of the baseline DA signal were not different between the rats repeatedly treated with nicotine and the saline control rats.

The ability of a challenge dose of nicotine to alter DA clearance in striatum in rats either repeatedly injected with nicotine or saline was determined and the data for each parameter (A_{max} , T_{80} and CL_{DA}) analyzed using separate three-way ANOVAs. With respect to A_{max} , a significant interaction of pretreatment x

challenge x time was found ($F_{13,403} = 2.22$, $p < 0.01$). Analysis of the data from the saline control group revealed that compared to baseline, A_{\max} gradually decreased by ~10% during the 60-min period, however no significant effect was observed (Figure 20, top panel). Thus, in the saline control group, DA application into striatum at 5-min intervals for 60 min did not alter A_{\max} .

To further analyze the interaction, subsequent between-groups one-way ANOVAs were conducted on the data from each 5-min time point from 10-60 min after injection (Figure 20, top panel). These analyses revealed that the nicotine control group (rats repeatedly injected with saline and challenged with nicotine on the experiment day) was significantly different ($p < 0.05$) from all of the other treatment groups from 10-35 min. Compared to the baseline response (within-subject comparisons), the onset of the nicotine-induced decrease in A_{\max} for the nicotine control group was 10 min after the nicotine challenge ($p < 0.05$), the duration of the decrease was from 10-35 min, and a maximal 36% decrease in A_{\max} was observed at the 30 min point. In contrast, within-subject comparisons also revealed that A_{\max} values did not differ from baseline across the 60 min session for the group injected with nicotine for 5 days followed by the nicotine challenge. Furthermore, no differences were observed in A_{\max} values between the group repeatedly injected with nicotine then challenged with nicotine and the saline control group. Taken together, these results indicate that repeated treatment with nicotine resulted in tolerance to the effect of nicotine to decrease A_{\max} .

In contrast to the effect of nicotine on A_{\max} , no significant main effects or interactions were observed for T_{80} and CL_{DA} , when the data were analyzed by three-way ANOVA (Fig 20, middle and bottom panels, respectively). CL_{DA} in the nicotine control group tended to increase, but this effect did not reach significance. Thus, compared to T_{80} and CL_{DA} , A_{\max} appears to be the more sensitive parameter for detection of the effect of repeated nicotine on clearance of exogenous DA in striatum.

IV. Discussion

The results from the current *in vivo* voltammetry study demonstrate that tolerance develops to the nicotine-induced increase in clearance of exogenous DA in MPFC and striatum. Following repeated injection with saline, challenge with nicotine resulted in an increase in DA clearance in MPFC and striatum consistent with previous findings (Middleton et al., 2004). The current study shows that in both MPFC and striatum, DA clearance was decreased in the group repeatedly administered nicotine and then challenged with nicotine on the experimental day compared to the nicotine control group, which was repeatedly administered saline and challenged with nicotine. Furthermore, DA clearance in the group repeatedly administered nicotine and then challenged with nicotine was not different from the saline control group, which was repeatedly administered saline and then challenged with saline. The data obtained from the CL_{DA} parameter is more variable than the A_{\max} parameter. This is likely due to

the fact that CL_{DA} takes into account multiple measurements over time, whereas, A_{max} only measure a single point. Thus, tolerance develops to the nicotine-induced increase in exogenous DA clearance in both MPFC and striatum. The current results extend previous findings showing that nicotine acutely increases DA clearance in the MPFC, striatum, and nucleus accumbens and that this effect is mediated by nicotinic receptors (Hart and Ksir, 1996; Middleton et al., 2004).

Previously, exogenous DA clearance has been defined as A_{max}/T_{80} with units of concentration/time (Zahniser et al., 1999). In more recent assessments of DAT function by this group (Sabeti et al., 2002), another parameter has been elaborated called DA clearance efficiency. DA clearance efficiency has been defined as a rate constant k , with units of s^{-1} and is determined by fitting the DA signal to a first-order exponential decay function, i.e., $A(t)=A_{max}*e^{-k(t-t_0)}$ (where A is the signal amplitude (μM) at any time t (s) following peak signal amplitude (A_{max}), and k is the first-order rate constant of decay of the DA signal). However, the classical physiological measure of clearance is not represented by either A_{max}/T_{80} (DA clearance) or k (DA clearance efficiency). In the current study, the classical pharmacokinetic approach has been utilized to assess exogenous DA clearance (Ito et al., 1998; Shargel et al., 2005). The parameter determined in the current study, CL_{DA} , was calculated as the amount of DA pressure ejected divided by the AUC for each DA signal, with units of volume/time. CL_{DA} can be directly equated to the physiological process of DA elimination from the ejection site and provides a direct measure of DA transport via DAT. Importantly, CL_{DA} is

independent of dose or volume of DA pressure ejected and is independent of the volume into which DA distributes under linear conditions. Thus, this analysis directly measures the efficiency of DA removal after its exogenous application, i.e., the transport process via DAT.

Nicotinic receptor activation acutely results in exocytotic DA release and an increase in the clearance of DA from the extracellular space. The increase in DA clearance may result in a sharpening of the DA signal. In both *in vitro* superfusion assays and *in vivo* microdialysis studies, nicotine-evoked DA release striatum and prefrontal cortex was reported to be greater following repeated systemic administration of nicotine than that in rats repeatedly injected with saline (Yu and Wecker, 1994; Nisell et al., 1996; Marshall et al., 1997). However, the role that DAT may play in the increase in extracellular DA levels following repeated nicotine was not considered in the previous studies. Previous research shows that nicotine acutely increases DA clearance, in MPFC, striatum, and nucleus accumbens (Hart and Ksir, 1996; Middleton et al., 2004), which would result in decreased extracellular DA concentrations. The current study shows that tolerance develops to the increase in DAT function (DA clearance) in MPFC and striatum following repeated nicotine administration. Thus, diminished DAT function as a result of repeated nicotine administration would be expected to result in greater extracellular DA concentrations consistent with the results of previous *in vitro* superfusion and *in vivo* microdialysis studies (Yu and Wecker, 1994; Marshall et al., 1997). The greater concentrations of DA in the

extracellular space may contribute to the salience of the environmental cues associated with chronic tobacco use, resulting in an enhancement of conditioned reward, making smoking cessation difficult.

Nicotinic receptor activation may modulate DAT function by alterations in DA reuptake into the presynaptic terminal and/or by altering DA efflux through the transporter under certain conditions. Nicotine has been reported to enhance amphetamine-evoked [³H]DA release from MPFC slices incubated in assay buffer in the absence of calcium (Drew et al., 2000). The absence of calcium in the assay buffer in the latter study precludes nicotine-evoked exocytotic DA release and suggest that nicotine-induced augmentation of the response to amphetamine involves DAT. The observed nicotine enhancement of the effect of amphetamine was inhibited by nicotinic receptor antagonists, mecamylamine and dihydro- β -erythroidine, but not by α -bungarotoxin, indicating the involvement of heteromeric nicotinic receptors. Interestingly, nicotine did not enhance amphetamine-evoked [³H]DA release in striatum or nucleus accumbens (Drew et al., 2000). Furthermore, the nicotine-induced enhancement of amphetamine-evoked [³H]DA release from MPFC was maintained after 10 days of nicotine administration (Drew and Werling, 2003). Thus, these findings suggest that nicotinic receptors also modulate DAT function to enhance amphetamine-stimulated reverse transport of DA by DAT, although in contrast to the current study, tolerance did not develop to the nicotinic receptor enhancement of reverse transport of DA by DAT.

One mechanism by which nicotine may modulate DAT function is via nicotinic receptor-induced augmentation of DAT trafficking to the presynaptic terminal membrane. The relatively rapid nicotine-induced increase in DA clearance suggests that new synthesis of DAT protein is not responsible. Rather, the time course of response is consistent with trafficking of intracellular stores of DAT protein to the terminal membrane. One potential explanation could involve the activation of second messenger signaling cascades. A study in hippocampal neurons revealed that nicotinic receptor activation resulted in an increase in intracellular calcium, activating mitogen-activated protein (MAP) kinase and calcium/calmodulin-dependent protein kinase pathways to activate the cyclic AMP response element binding protein producing transcriptional effects (Hu et al., 2002). Inhibition of the MAP kinase pathway results in trafficking of DAT from the plasma membrane to intracellular pools measured by biotinylation assays conducted in human embryonic kidney 293 cells (Morón et al., 2003). Taken together, these data suggest a potential role of the MAP kinase pathway to increase DAT localization on the plasma membrane, which could increase DA clearance following acute administration of nicotine. Repeated nicotine may result in an inhibition of the MAP kinase pathway resulting in trafficking of DAT from the plasma membrane, resulting in tolerance to the nicotine-induced increase in DA clearance.

Tolerance to chronic nicotine administration has been shown in various animal models, i.e., locomotor activity, striatal dopamine metabolism and of nicotine-induced antinociception (Stolerman et al., 1973; Marks et al., 1983; Marks et al., 1991; McCallum et al., 2000; Pietila and Ahtee, 2000). Another potential explanation for the observed tolerance to the nicotine-induced enhancement of DA clearance is nicotinic receptor desensitization. Chronic nicotine application resulted in an upregulation of $\alpha 4\beta 2$ receptors in *Xenopus* oocytes (Fenster et al., 1999). Furthermore, the EC_{50} for nicotine to produce desensitization was the same as that required for upregulation, suggesting that upregulation occurs as a result of desensitization. Using various doses of nicotine and different dosing regimens, Rowell and Li (1997) showed that nicotinic receptor desensitization was dependent on dose and regimen of administration. Concentrations of nicotine that are achieved by smokers desensitize nicotinic receptors located on the mesolimbic DA neurons (Pidoplichko et al., 1997). Tolerance following chronic nicotine has been shown in the absence of upregulation of nicotinic receptors (Pauly et al., 1992; McCallum et al., 2000). However, it is also possible that the observed tolerance is due to nicotinic receptor upregulation. Mice chronically treated with nicotine have shown an increase in nicotine binding sites in brain (Marks et al., 1983; Schwartz and Kellar 1983; Collins et al., 1988; Kassiou et al., 2001; Nuutinen et al., 2005). Recently, in $\beta 2$ knockout mice, following chronic nicotine treatment, upregulation of nicotine binding sites is no longer observed (McCallum et al.,

2005). Therefore, nicotine may have differential effects on DAT function following repeated administration compared to acute nicotine administration.

More than one nicotinic receptor subtype is thought to be located on DA neurons. Neurotoxins (α -Conotoxin MII) as well as small molecules have been shown to inhibit only 50% of nicotine evoked [3 H]DA release, suggesting the involvement of more than one nicotinic receptor subtype in the DA releasing properties of nicotine (Kulak et al., 1997; Kaiser et al., 1998; Wilkins et al., 2002; Grinevich et al., 2003; Wei et al., 2005). Furthermore, nicotinic receptor subtypes also desensitize at different rates, i.e., $\alpha 7$ desensitizes more rapidly than $\alpha 4\beta 2$ (Fenster et al., 1997). It is possible that the subtypes mediating DA release and those modulating DAT function desensitize differently. Therefore, following repeated nicotine treatment the nicotinic receptors modulating exocytotic DA release may not desensitize leading to an increase in DA release, while the nicotinic receptors modulating DAT may desensitize resulting in tolerance to the nicotine-induced increase in DA clearance. Together these mechanisms would result in an increase in the extracellular DA concentration. Thus, chronic nicotine is enhancing DA release and concomitantly decreasing DA clearance (current study), which may be an important mechanism involved in the maintenance of smoking behavior.

Finally, tolerance to the nicotine-induced increase in DA clearance may be due to an increase in nornicotine production. Nornicotine decreases DAT function (Chapter Three). Following systemic nicotine injection, nicotine reaches maximal brain concentration within 5 min and has a brain $t_{1/2}$ of 52 min. However, nicotine metabolism results in production of nornicotine (Ghosheh et al., 1999). Following nicotine injection nornicotine reaches maximal brain concentrations 60 min following nicotine injection and has a brain $t_{1/2}$ of 166 min (Ghosheh et al., 1999). Furthermore, following repeated nicotine treatment, nornicotine accumulates in brain (Ghosheh et al., 2001). Therefore, it is possible the nicotine is no longer able to increase DAT function following repeated nicotine treatment because nornicotine is inhibiting DAT function.

In summary, the current study shows that tolerance develops to nicotine-induced enhancement of DAT function following repeated nicotine administration. Tolerance to the nicotine-induced increase in DA clearance may be important to our understanding of the rewarding properties of tobacco use. Further studies are warranted to examine the pathways involved in the modulation of DAT function following both acute and repeated nicotine administration.

Figure 18. Representative baseline DA signal in MPFC of urethane-anesthetized rats prior to nicotine or saline challenge. Electrochemical signals were obtained following pressure ejection of DA (200 μM) and represent the baseline response. Three parameters are obtained from each DA signal; A_{max} , T_{80} and CL_{DA} . Data are expressed as μM DA as a function of time (s) after DA pressure ejection. DA concentrations were calculated based on calibration curves generated *in vitro*.

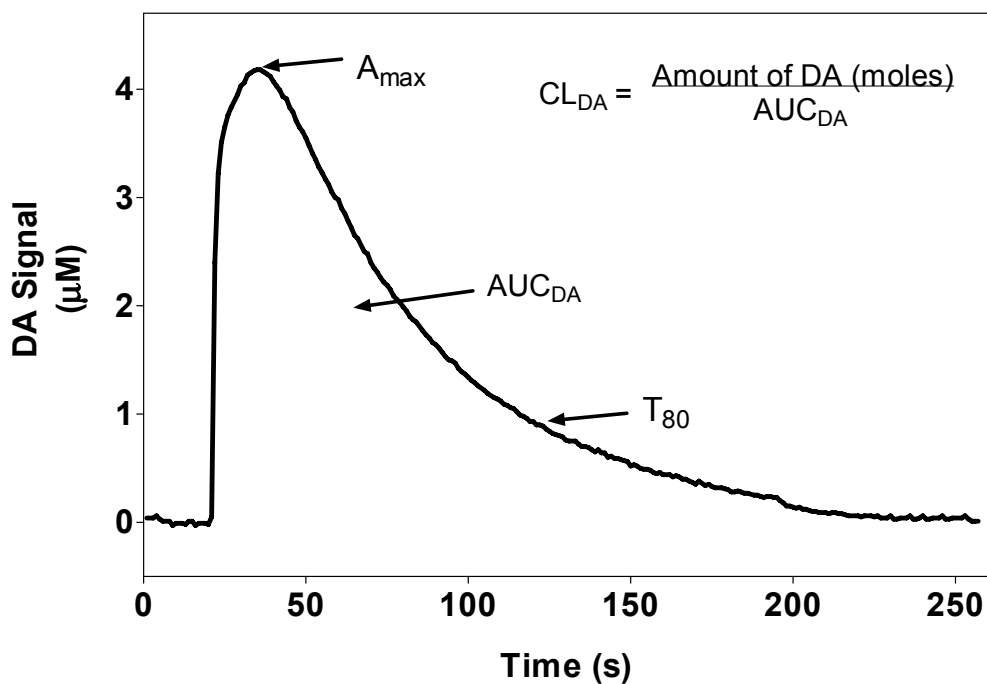


Figure 19. Tolerance develops to the nicotine-induced decrease in A_{\max} and increase in CL_{DA} in MPFC. A_{\max} , T_{80} and CL_{DA} are illustrated in the top, middle and bottom panels, respectively. Rats were injected (s.c.) with nicotine (0.4 mg/kg) or saline once daily for 5 days; 24 hr after the last injection, rats were anesthetized and exogenous DA clearance determined using *in vivo* voltammetry. After stable baseline signals were obtained in response to pressure ejection of 200 μ M DA at 5-min intervals, rats were challenged with nicotine (0.4 mg/kg) or saline and exogenous DA applied at 5-min intervals for 60 min. Arrow indicates the time point that rats received the challenge injection. Data are expressed as mean and S.E.M. as a percentage of the respective baseline values as a function of time (min). Baseline values for each parameter are provided in the Results section. Legend indicates treatment groups, i.e., repeated injection - challenge injection. SAL-SAL indicates the saline control group; SAL-NIC indicates the nicotine control group; NIC-SAL indicates the group repeatedly administered nicotine followed by saline challenge; NIC-NIC indicates the group repeatedly administered nicotine followed by nicotine challenge (n = 8 – 10 rats/group). # difference compared to baseline for the SAL-SAL group; * difference between SAL-NIC group and all other groups.

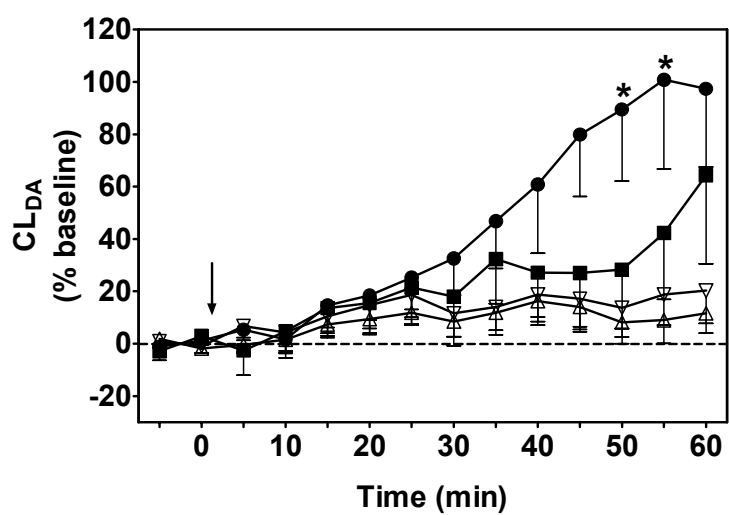
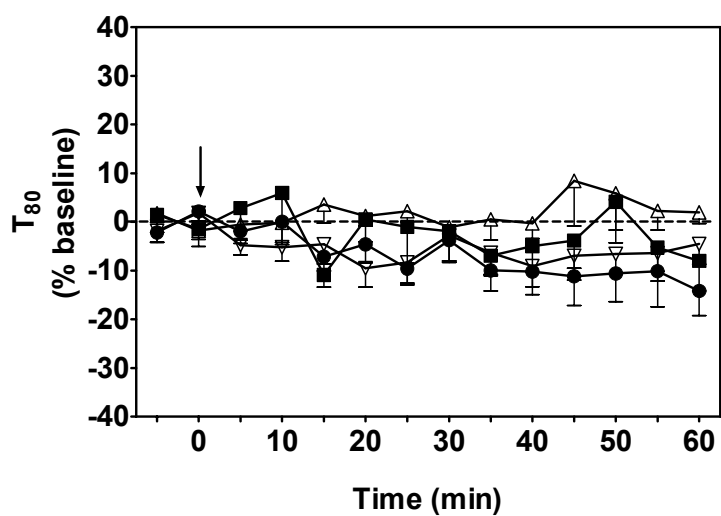
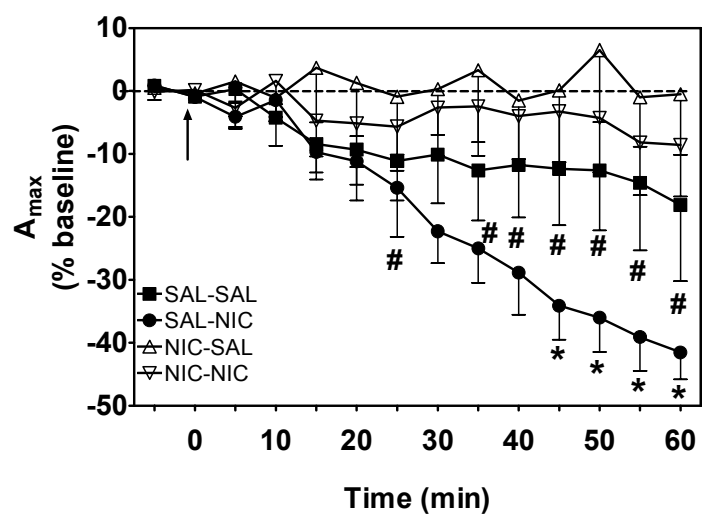
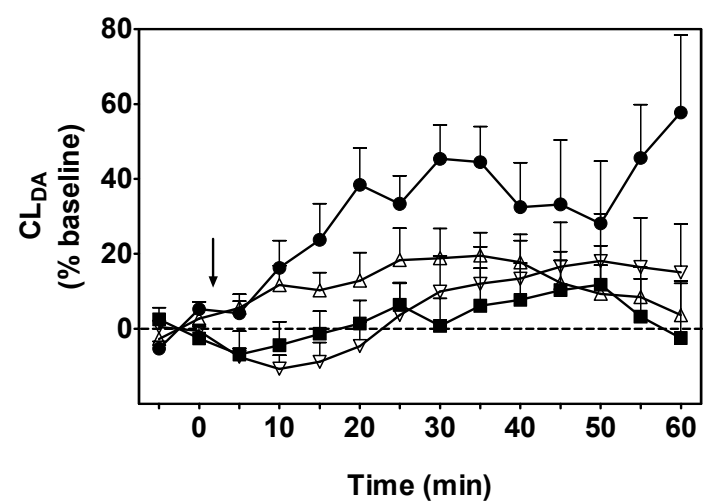
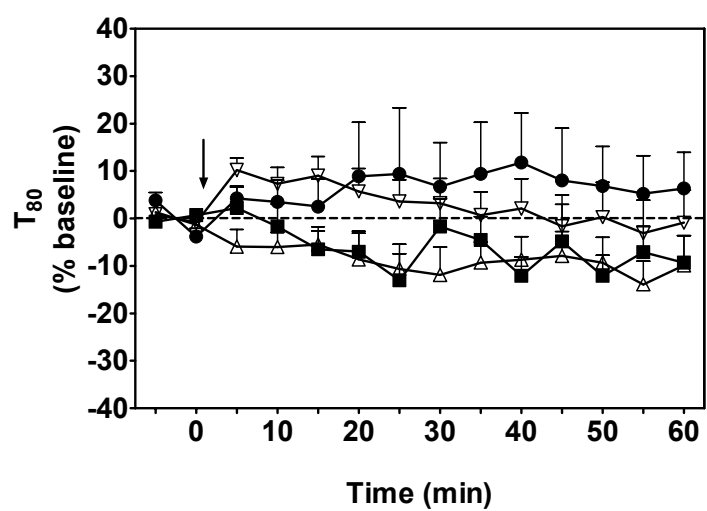
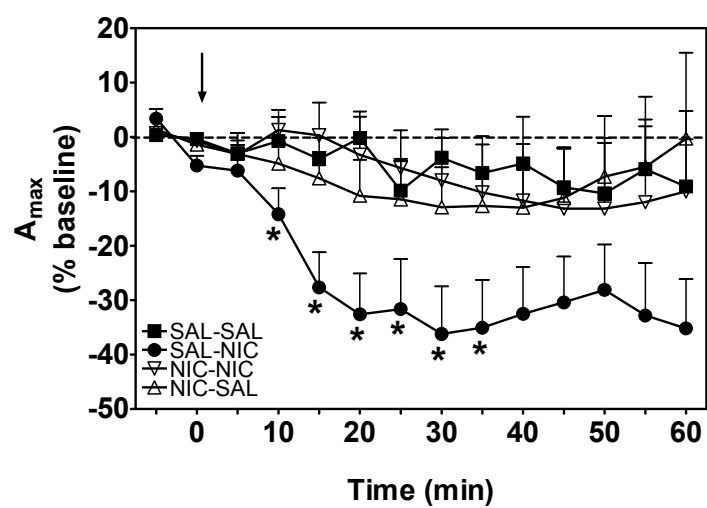


Figure 20. Tolerance develops to the nicotine-induced decrease in A_{\max} and increase in CL_{DA} in striatum. A_{\max} , T_{80} and CL_{DA} are illustrated in the top, middle and bottom panels, respectively. Experiments were conducted as described in Figure 19, except that the nicotine dose was 0.8 mg/kg and exogenous DA clearance was determined in striatum. Baseline values for each parameter are indicated in the Results section. Legend is as described in Figure 19. (n = 8 – 10 rats/group). Arrow indicates the time point that rats received the challenge injection. * difference between SAL-NIC group and all other groups.



Chapter Five

Nicotine Increases Dopamine Transporter Function via a Trafficking-Independent Mechanism

I. Introduction

Nicotine is generally accepted to be the alkaloid in tobacco primarily responsible for nicotine dependence (Clarke, 1987; Pomerleau and Pomerleau, 1992). Activation of nicotinic receptors by nicotine results in an increase in the extracellular concentration of DA, which is thought to mediate the rewarding effects of nicotine and maintain tobacco use in dependent individuals (Corrigall et al., 1992; Koob, 1992; Stolerman and Jarvis, 1995). Acute nicotine increases DA release from its presynaptic terminals in the striatum in a concentration-dependent manner (Andersson et al., 1981; Dwoskin et al., 1999; Kaiser et al., 1998; Ksir et al., 1995; Pontieri et al., 1996; Rowell et al., 1987; Vezina et al., 1992; Westfall et al., 1983). Synaptic DA concentrations are regulated by the DAT, which transports extracellular DA into the presynaptic terminal. DAT is a major target for psychostimulants, e.g., cocaine and amphetamine (Horn, 1990). DAT has been shown to be regulated by constitutive internalization and recycling (i.e., trafficking), involving transporter phosphorylation and protein-protein interactions (Kahlig and Galli, 2003; Loder and Melikian, 2003; Melikian, 2004; Torres et al., 2003; Zahniser and Doolen, 2001).

Psychostimulants and second messengers alter DAT function and trafficking (Garcia et al., 2005; Gnegy et al., 2004; Kahlig et al., 2004; Holton et

al., 2005; Sorkina et al., 2005). For example, cocaine and amphetamine act at DAT to decrease DA clearance in *in vivo* voltammetry studies (Cass et al., 1993; Zahniser et al., 1999). In contrast, nicotine has been shown to increase the clearance of exogenously applied DA in rat striatum, nucleus accumbens and medial prefrontal cortex in *in vivo* voltammetry studies (Hart and Ksir, 1996; Middleton et al., 2004). DAT surface expression in cell expression systems is also acutely sensitive to amphetamine and cocaine, which decreases and increases DAT surface levels, respectively (Daws et al., 2002; Little et al., 2002; Saunders et al., 2000; Zahniser and Sorkin, 2004). Although through different mechanisms, nicotine and amphetamine both evoke [³H]DA release from superfused rat striatal slices (Corrigall et al., 1992; Koob, 1992; Stolerman and Jarvis, 1995). Previous results shows that nicotine does not alter [³H]DA uptake into striatal synaptosomes upon *in vitro* exposure to nicotine (Carr et al., 1989; Zhu et al., 2003). However, the effect of nicotine on [³H]DA uptake into striatal synaptosomes and on DAT trafficking has not been determined following systemic nicotine administration.

Thus, the present study determined if nicotine administration altered [³H]DA uptake into rat striatal synaptosomes and the cellular localization of DAT in striatum using cell surface biotinylation and subfractionation approaches. The ability of nicotine to modulate DAT function, and thereby extracellular DA concentration, may have physiological importance with regards to nicotine enhancement of cognitive processes such as attention, learning and memory, as

well as important clinical relevance with respect to schizophrenia and drug abuse. Therefore, understanding nicotine-induced regulation of DAT function may provide further insights into the mechanisms of nicotine action.

The current series of experiments will test the hypotheses that 1) systemic pretreatment with nicotine will increase DAT function in striatal synaptosomes in vitro, similar to the increase in DA clearance observed in striatum in vivo, 2) the effect of nicotine to increase the Vmax of [³H]DA uptake in striatum is mediated by nicotinic receptors located on DA nerve terminals in striatum, 3) systemic administration of nicotine will increase the number of DAT sites in striatum as measured by radioligand binding, and 4) systemic administration of nicotine will increase the trafficking of DAT to the neuronal cell surface in striatum.

II. Methods

Ila. Materials. Antibodies recognizing rat DAT (sc-1433; goat polyclonal antibody), calnexin (sc-11397; rabbit polyclonal antibody); β -actin (sc-7210; rabbit polyclonal antibody) and protein phosphatase 2A (PP2A; sc-6110; goat polyclonal antibody) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-goat antibody was purchased from Dako Cytomation (#Z0454; Carpinteria, CA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody was purchased from Bio-Rad (Hercules, CA). GBR 12909, nicotine ditartrate and nomifensine maleate were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfosuccinimidobiotin (sulfo-NHS-biotin) and

immunoPure immobilized monomeric avidin gel were purchased from Pierce Biotechnology, Inc. (Rockford, IL). [^3H]Dopamine (3,4-ethyl-2 [N - ^3H] dihydroxyphenylethylamine; specific activity 31 Ci/mmol) and [^3H]GBR 12935 ([Propylene-2,3- ^3H] (1-[2-diphenylmethoxy)ethyl]-4-(3-phenylpropyl)-piperazine); specific activity 43.5 Ci/mmol) were purchased from New England Nuclear (Boston, MA). D-Glucose was purchased from Aldrich Chemical Co, Inc. (Milwaukee, WI). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

IIb. Animals. Male Sprague-Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN) and were housed with free access to food and water in a colony room in the Division of Laboratory Animal Resources in the College of Pharmacy at the University of Kentucky. Animal handling procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky and were performed in accordance with the 1996 version of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

IIc. Synaptosomal [^3H]DA uptake. [^3H]DA uptake assays were conducted using a previously published method (Zhu et al. 2003). Separate groups of rats were injected with nicotine (0.8 mg/kg, s.c.) or saline. Nicotine dose was chosen based on previous studies from our laboratory showing that nicotine (0.32 mg/kg, s.c.) increased DA clearance (~60%) in striatum *in vivo*

(Middleton et al., 2004). In the current study, rats were killed 5, 10, 40 or 60 min post-injection. Striata were homogenized in 20 ml of ice-cold 0.32 M sucrose solution containing 5 mM sodium bicarbonate (pH 7.4) with 16 passes of a Teflon pestle homogenizer (clearance, 0.015 in). Homogenates were centrifuged (2,000 *g*, 4 °C, 10 min), and resulting supernatants were centrifuged (20,000 *g*, 4 °C, 15 min). Resulting pellets were resuspended in 2.4 ml of ice-cold assay buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgSO₄, 1.25 mM CaCl₂, 1.5 mM KH₂PO₄, 10 mM glucose, 25 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), 0.1 mM EDTA, 0.1 mM pargyline and 0.1 mM L-ascorbic acid, saturated with 95% O₂/5% CO₂, pH 7.4). Striatal synaptosomes (20 µg protein in 50 µl) were incubated in an oxygenated environment for 5 min at 34 °C. Subsequently, one of ten [³H]DA concentrations (1 nM - 5 µM) were added to each tube. Total assay volume was 500 µl. Nonspecific [³H]DA uptake was determined in the presence of 10 µM nomifensine. Incubation continued for 10 min at 34 °C and was terminated by the addition of 3 ml of ice-cold assay buffer containing pyrocatechol (1 mM), followed by immediate filtration through Whatman GF/B glass fiber filters (presoaked with 1 mM pyrocatechol for 3 h). Filters were washed 3 times with 3 ml ice-cold buffer containing 1 mM pyrocatechol using a Brandel cell harvester (Model MP-43RS; Biochemical Research and Development Laboratories Inc., Gaithersburg, MD). Radioactivity was determined by liquid scintillation spectrometry (Model B1600TR, Perkin-Elmer Life Sciences, Downers Grove, IL). Protein concentrations were determined with bovine serum albumin as the standard (Bradford, 1976). V_{\max}

and K_t were determined using GraphPad Prism software (GraphPad Prism, version 3.0; GraphPad Software, San Diego, CA).

IId. [^3H]GBR 12935 Binding. To determine if nicotine pretreatment alters the total number of DAT sites, [^3H]GBR 12935 binding assays were performed using striata obtained from groups of rats injected with nicotine (0.8 mg/kg, s.c.) or saline and killed 5, 10 or 40 min post-injection. Striata was obtained and stored at $-70\text{ }^{\circ}\text{C}$ until assay. Striata were homogenized with a polytron homogenizer (setting 40; Tekmar, Cincinnati, OH), in 10 volumes of ice-cold assay buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl_2 , and 1.2 mM MgSO_4 , pH 7.5). Homogenates were incubated at $37\text{ }^{\circ}\text{C}$ for 5 min, placed on ice, and centrifuged (25,000 g , 20 min, $4\text{ }^{\circ}\text{C}$). Pellets were resuspended in 10 volumes of ice-cold Milli-Q water, incubated at $37\text{ }^{\circ}\text{C}$ for 5 min, and centrifuged (25,000 g , 20 min, $4\text{ }^{\circ}\text{C}$). Pellets were resuspended in 10 volumes of ice-cold 10% assay buffer and incubation and centrifugation steps repeated twice. Final pellets were stored in 10% assay buffer at $-70\text{ }^{\circ}\text{C}$ until assay, where upon pellets were resuspended in 20 ml assay buffer. Samples (250 μl), consisting of 100 – 140 μg of membrane protein and a range of [^3H]GBR 12935 concentrations (0.1–35 nM) in assay buffer containing 50 mM Tris, were incubated for 90 min at $4\text{ }^{\circ}\text{C}$. Nonspecific binding was determined in the presence of 10 μM GBR 12909. Reactions were terminated by dilution of the samples with 3 ml of ice-cold 20 mM Krebs-HEPES buffer followed by immediate filtration through Whatman GF/B glass fiber filters presoaked for 2 hrs in 0.5% polyethylenimine using the Brandel

harvester. Filters were rinsed 3 times with 3 ml of ice-cold 20 mM buffer, transferred to vials, and scintillation cocktail (4 ml) added. Radioactivity was determined using a Tri-Carb 2100 TR liquid scintillation analyzer (PerkinElmer Life Sciences). Protein concentrations were determined as previously described. Kinetic parameters (B_{\max} and K_d) of [^3H]GBR 12935 binding were determined using GraphPad Prism software, version 3.0.

Ile. Biotinylation and Immunoblotting Assay. For the determination of total, surface and intracellular levels of DAT protein in striatal synaptosomes, surface biotinylation and immunoblot analysis were performed as described previously (Apparsundaram et al., 1998; Melikian et al., 1996; Ramamoorthy et al., 1998; Zhu et al., 2005). Striatal synaptosomes were obtained as described above for [^3H]DA uptake assays. Impermeant biotinylation reagent sulfo-NHS-biotin was used for the isolation of plasma membrane proteins. DAT protein was identified using polyclonal DAT antibody (Taubenblatt et al., 1999; Salvatore et al., 2003; Vaughan et al., 1997).

Specifically, samples of striatal synaptosomes (500 μg total protein) were incubated for 1 h at 4 °C with continual shaking in 500 μl of 1.5 mg/ml sulfo-NHS-biotin in phosphate buffered saline/Ca/Mg buffer (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 9.6 mM Na_2HPO_4 , 1 mM MgCl_2 , 0.1 mM CaCl_2 , pH 7.3). After incubation, samples were centrifuged (8,000 g , 4 °C, 4 min). To remove the free biotinylation reagent, the resulting pellet was resuspended in 1 ml of ice-cold 100

mM glycine in PBS/Ca/Mg buffer and centrifuged (8,000 *g*, 4 °C, 4 min). The resuspension and centrifugation steps were repeated. Final pellets were resuspended in 1 ml of ice-cold 100 mM glycine in PBS/Ca/Mg buffer and incubated with continual shaking for 30 min at 4 °C. Samples were centrifuged (8,000 *g*, 4 °C, 4 min) and the resulting pellets were resuspended in 1 ml ice-cold PBS/Ca/Mg buffer and centrifuged again. Resuspension and centrifugation steps were repeated twice to remove excess glycine. Final pellets were lysed by sonication for 2-4 s in 300 µl Triton X-100 buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µM pepstatin, 250 µM phenylmethanesulfonyl fluoride) followed by incubation and continual shaking for 20 min at 4 °C. Lysates (300 µl) were centrifuged (21,000 *g*, 4 °C, 20 min). Pellets were discarded and 100 µl of each supernatant was stored at -20 °C for determination of total immunoreactive DAT. Remaining supernatant was incubated with continuous shaking in the presence of monomeric avidin beads in Triton-X100 buffer (100 µl/tube) for 1 h at 22-24 °C. Samples were centrifuged (17,000 *g*, 4 min, 4 °C), and supernatants containing non-biotinylated proteins (intracellular) were stored at -20 °C. Resulting pellets containing biotinylated proteins (cell-surface) were resuspended in 1 ml of 1.0% Triton X-100 buffer and centrifuged (17,000 *g*, 4 min, 4 °C), and the pellets were resuspended and centrifuged two times. Final pellets consisted of biotinylated proteins adsorbed to monomeric avidin beads. Biotinylated proteins were eluted by incubating with 50 µl Laemmli buffer (62.5 mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.05% β-mercaptoethanol and 0.05%

bromophenol blue, pH 6.8) for 20 min at 22-24 °C. Total, intracellular and cell surface fractions were stored at -20 °C.

Samples (total, intracellular and cell surface fractions) were thawed and subjected to gel electrophoresis and Western blotting as previously described (Melikian et al. 1994; Salvatore et al. 2003; Zhu et al., 2005). Briefly, proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min at 150 V and transferred to Immobilon-P transfer membranes (Cat # IPVH00010, 0.45 µm pore size; MILLIPORE Co., Bedford, MA) in transfer buffer (50 mM Tris, 250 mM glycine, 3.5 mM SDS) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Ltd., Hercules, CA) for 110 min at 72 V. Transfer membranes were incubated with blocking buffer (5% milk powder in PBS containing 0.5% Tween 20) for 1 h at 22-24 °C, followed by incubation with goat polyclonal DAT antibody (1 µg/ml in blocking buffer) overnight at 4 °C. Transfer membranes were washed 5 times with wash buffer (PBS containing 0.5% Tween 20) at 22-24 °C, and then incubated with rabbit anti-goat antibody (1:2500 dilution in blocking buffer) for 1 h at 22-24 °C. Transfer membranes were then washed and incubated with peroxidase-conjugated goat anti-rabbit antibody (diluted 1:5000) for 1 h at 22-24 °C. Protein bands were detected using enhanced chemiluminescence and developed on Hyperfilm (ECL-plus; Amersham Biosciences UK Ltd., Little Chalfont Buckinghamshire, UK). After detection and quantification of DAT protein, each blot was stripped using Tris buffer (62.5 mM Tris-HCl with 2% SDS and 100 mM β-mercaptoethanol, pH 6.8) and reprobbed for detection of PP2A and calnexin.

PP2A, an intracellular protein (Janssens and Goris, 2001), served as a control protein to monitor the efficiency of biotinylation of cell surface proteins and was detected using goat polyclonal PP2A antibody (1:500). Calnexin, an endoplasmic reticular protein (Hochstenbach et al., 1992; Krijnse-Locker et al., 1995; Rajagopalan et al., 1994), was detected using rabbit polyclonal calnexin antibody (1:5000) to monitor biotinylation of intracellular proteins. β -Actin was quantified to normalize for protein loading across samples.

Immunoreactive bands were quantified by densitometric scanning using Scion image software (Scion Corp., Frederick, MD). Band density measurements were used to calculate levels of DAT in total, non-biotinylated and biotinylated fractions. Specifically, total DAT levels were calculated based on the density of DAT-immunoreactive bands in an aliquot of synaptosomal extract multiplied by the total volume of extract and divided by the total volume of synaptosomal extract subjected to SDS-PAGE. DAT levels in the non-biotinylated fractions were calculated as density of DAT-immunoreactive bands in an aliquot of supernatant post-avidin incubation multiplied by the total volume of the extract and divided by the volume of supernatant subjected to SDS-PAGE. In preliminary studies, quantification of PP2A revealed a maximum 10% contamination of intracellular proteins in the plasma membrane biotinylated fraction. Immunoreactive bands were quantified and were within the linear range of detection.

IIf. Subcellular Fractionation. To verify the results using the biotinylation approach, a subcellular fractionation strategy was adapted from previously described methods using presynaptic vesicular proteins (Clift-O'Grady et al., 1990; Huttner et al., 1983). Briefly, rat striata were homogenized in 0.32 M sucrose buffer containing 5 mM HEPES-NaOH (pH 7.3) using a Wheaton Instruments Potter Elvehjem homogenizer (10 strokes) and centrifuged (1000 *g*, 10 min, 4°C). Supernatants were centrifuged (13,000 *g*, 17 min, 4°C) to yield a crude synaptosomal pellet (P2). Synaptosomes in this P2 fraction (1 mg) were lysed by homogenization (5 strokes) in ice-cold 5 mM HEPES-NaOH (pH 7.4) plus protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µM pepstatin, 250 µM phenylmethanesulfonyl fluoride). Synaptic plasma membranes (LP1) and other large membranes were separated at 15,000 *g* for 20 min. The vesicle enriched LP2 pellet was obtained following centrifugation of the resulting supernatant (LS1 Fraction; 200,000 *g*, 30 min, 4°C). Proteins were extracted from each fraction with 1% SDS, 5 mM HEPES-KOH (pH 7.3), 1 mM EDTA, 1 mM EGTA and protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µM pepstatin, 250 µM phenylmethanesulfonyl fluoride). Protein concentrations were determined using the bicinchoninic acid method (Pierce, Rockford, IL). Each fraction (50 µg) was subjected to immunoblot analysis with antibodies against DAT, PP2A, calnexin and β-actin. DAT-immunoreactive bands were detected using goat polyclonal anti-DAT antibody (1 µg/ml) in blocking buffer (5% milk in PBS containing 0.05% Tween 20, pH 7.4) followed by rabbit polyclonal anti-goat antibody (1:2500 dilution in blocking buffer) and goat alkaline phosphatase conjugated polyclonal

anti-rabbit antibody (1:10,000 dilution). Protein bands present in the transfer membrane were measured using enhanced chemifluorescence (GE Healthcare, Piscataway, NJ) with a Typhoon Imaging System (GE Healthcare) and Imagequant TL software (GE Healthcare). Immunoreactive bands were quantified and were within the linear range of detection. β -Actin was quantified to normalize for protein loading across samples. Relative proportion of DAT in the plasma membrane was expressed by calculating the ratio LP1/LP1+LP2.

III. Results

IIIa. Nicotine administration increases the V_{\max} for [^3H]DA uptake in rat striatum. Kinetic analysis of [^3H]DA uptake was performed in synaptosomes obtained 5, 10, 40 or 60 min following administration of nicotine (0.8 mg/kg, s.c.) or saline. Synaptosomal [^3H]DA uptake was not different between the saline control groups across the time points and as such the data were pooled for statistical analysis and graphical presentation (Figure 21). While the interaction between treatment and time was not significantly different, a main effect of nicotine treatment was observed ($F_{1,48} = 4.16$; $p < 0.05$). Compared to the saline control group, nicotine significantly increased (~25%) the V_{\max} for [^3H]DA uptake at 10 and 40 min post-injection ($p < 0.05$). The effect of nicotine on V_{\max} was not significant at either the 5 or 60 min time points. There was no change in K_t at any time point (Table 2).

IIIb. Nicotine administration does not alter [³H]GBR 12935 binding to rat striatal membranes. No differences between nicotine (0.8 mg/kg) and saline groups were observed in either the B_{max} or K_d for [³H]GBR 12935 binding at the 5, 10 or 40 min time points (Table 3), suggesting that nicotine pretreatment does not alter the total amount of DAT protein in striatum.

IIIc. Nicotine administration does not alter the cellular localization of DAT in striatum. To determine if nicotine pretreatment (0.8 mg/kg) altered cell surface localization of DAT, rats were administered nicotine or saline and killed 10 or 40 min later. Striatal synaptosomes were prepared and biotinylation and subfractionation assays were performed. In all of the groups tested, DAT bands were observed at 80 kDa, as previously reported (Salvatore et al., 2003; Zhu et al., 2005). In biotinylation assays, DAT-immunoreactive bands were detectable in all three fractions. DAT band density was not different between nicotine and saline treated groups at the 10 and 40 min time points (Figure 22). There were no between-group differences in the levels of control proteins, PP2A (60 kDa), calnexin (90 kDa) and β-actin (42 kDa). Consistent with the results from the biotinylation assays, subfractionation experiments also revealed no difference in DAT levels in total and plasma membrane fractions (LP1). No differences between the nicotine and saline control groups were observed in the ratio of LP1/LP1+LP2 at the 10 min and 40 min time points (Figure 23). Also, there was no difference in the levels of control proteins, β-actin, PP2A and calnexin, between nicotine and saline control groups.

IV. Discussion

The current results demonstrate that 10 and 40 min following nicotine (0.8 mg/kg) administration, a significant increase (25%) in the V_{\max} for [^3H]DA uptake into striatal synaptosomes was observed. These data are consistent with previous findings from our laboratory showing that systemic nicotine increases DA clearance *in vivo* in rat striatum (Middleton et al., 2004). The increase in DA clearance observed *in vivo* is likely due to an increase in V_{\max} for DA. The nicotine-induced increase in [^3H]DA uptake into striatal synaptosomes was not accompanied by an increase in the total amount of DAT protein, as indicated by the lack of change in [^3H]GBR 12935 binding and in total DAT protein assessed by immunoblotting methods in comparison with the saline control group. Furthermore, the biotinylation and subfractionation results revealed no changes in the cellular distribution of DAT in striatum following nicotine administration. Taken together, these results suggest that nicotine increases striatal DA uptake *in vitro* and DA clearance *in vivo* via a trafficking-independent mechanism.

Previous research using *in vivo* voltammetry revealed that nicotine induced an increase in exogenous DA clearance in striatum in anesthetized rats (Middleton et al., 2004). The nicotine-induced increase in DA clearance was dose dependent with the 0.8 mg/kg dose increasing clearance by ~60%. Nicotine significantly increased DA clearance 15 min following nicotine administration and the increase persisted for one hour post injection (Middleton et al., 2004). Consistent with the latter study, results from the current study show

a 25% increase in V_{\max} for [^3H]DA uptake into striatal synaptosomes. However, the percent increase in the current study was lower than that observed in the *in vivo* voltammetry studies. Furthermore, DAT function in the current *in vitro* study was increased at 10 and 40 min post-injection and was back to saline control levels by 60 min following nicotine injection, whereas the effect of nicotine was still apparent at 60 min in the *in vivo* voltammetry studies. Differences in the magnitude and duration of nicotine effect on DAT function in the two assays may be due to the fact that DA clearance in the *in vivo* voltammetry assays is measured in localized areas of striatum, whereas the *in vitro* assay utilizes the entire striatum, potentially diluting localized changes. In this regard, results from *in vivo* voltammetry studies show that the striatum is heterogeneous with respect to DA clearance, which may be related to variations in the density DAT (Ciliax et al., 1995). Thus, the smaller magnitude of nicotine effect in the synaptosomal preparation may be due to an averaging of effect across this heterogeneous brain region. Furthermore, differences between the assays, including use of anesthetic and repeated exposure to exogenous DA in the *in vivo* studies, may have contributed to the observed differences in the magnitude and duration of the effect of nicotine on DAT function.

Although *in vivo* nicotine administration results in an increase in striatal DAT function as demonstrated using *in vivo* voltammetry and *in vitro* synaptosomal [^3H]DA uptake, previous results show that when striatum is exposed to nicotine *in vitro*, nicotine produces no effect on [^3H]DA uptake (Carr

et al., 1989; Zhu et al., 2003). These contrasting findings may be due to the effect of nicotine at the dopaminergic cell body or at circuitry which has been disrupted during the preparation of striatal slice or synaptosomes used in the *in vitro* studies.

Several studies have shown that DAT undergoes internalization and recycling, which may involve dynamin-clathrin mediated pathways and multiple protein-protein interactions, such as syntaxin-1A, PP2A, PICK1 and synuclein (Lee et al. 2004; Melikian, 2004; Torres et al., 2003; Zahniser and Doolen, 2001). Changes in DAT surface expression have been shown to be induced by psychostimulants or protein kinase C activation, and such changes correlate with alterations in [³H]DA uptake in striatum (Chi and Reith, 2003; Copeland et al. 1996; Vaughan et al., 1997) and in cell systems expressing DAT (Kahlig et al., 2004; Little et al., 2002; Pristupa et al. 1998). The previous studies used biotinylation and subfractionation approaches to show drug-induced changes in DAT cellular localization. One mechanism by which the nicotine-induced increase in DAT function may occur is *via* the trafficking of DAT to the cell surface. To assess this potential mechanism, cell surface biotinylation and subfractionation approaches were employed to assess DAT cellular localization in striatum. Recent studies have used the biotinylation approach for determination of DAT distribution in total, plasma membrane and intracellular fractions in rat striatal synaptosomes (Chi and Reith, 2003; Salvatore et al., 2003; Zhu et al., 2005). In the current study, results were also verified using the

subfractionation approach for the determination of DAT localization. However, the biotinylation assay revealed no differences in DAT cellular localization between the nicotine-treated and saline control groups; and, this finding was further confirmed using the subfractionation approach. Thus, it appears that the nicotine-induced increase in DAT function in striatum may occur via a trafficking-independent mechanism.

Alterations in transporter function in the absence of changes in transporter trafficking have been reported previously (Apparsundaram et al., 2001; Zhu et al., 2005). Specifically, insulin increases norepinephrine transporter function without a change in transporter cellular localization (Apparsundaram et al., 2001). The insulin-induced increase in NET function is likely due to activation of p38 MAP kinase and PI-3 kinase pathways (Apparsundaram et al., 2001). Similarly, p38 MAP kinase stimulation of the serotonin transporter was also recently shown to occur *via* a trafficking-independent mechanism (Zhu et al., 2005), suggesting that multiple pathways exist to regulate neurotransmitter transporter function.

Nicotine has been shown to activate several different second messenger pathways. For example, nicotine releases nitric oxide from rat hippocampal slices (Smith et al., 1998). Nicotine-induced nitric oxide release is inhibited by α -bungarotoxin, suggesting the involvement of Ca^{++} permeable $\alpha 7$ nicotinic receptors. Also, nicotinic receptor activation induces extracellular signal-regulated kinase phosphorylation in PC12 cells (Nakayama et al., 2001), as well

as alters calmodulin and mitogen-activated protein kinase function (Hu et al., 2002). Recently, the nitric oxide pathway has been implicated in increasing DAT function in striatum in studies using rotating disk electrode voltammetry (Volz and Schenk, 2004). It is possible that such signaling mechanisms may be involved in the nicotine-induced increase in DAT function without altering cell surface localization of DAT.

Figure 21. Nicotine administration increased the V_{\max} for [^3H]DA uptake in striatal synaptosomes. Rats were injected with nicotine (0.32 mg/kg, s.c.; open bars) or saline (control, CON; closed bar), and synaptosomes prepared 5, 10, 40 or 60 min post-injection. Specific [^3H]DA uptake for the 5, 10, 40 and 60 min saline control groups was 32.8 ± 1.66 , 29.7 ± 1.18 , 27.8 ± 1.45 , and 33.2 ± 1.72 , respectively. Nicotine increased the V_{\max} for [^3H]DA uptake 10 and 40 min following nicotine injection. Data are expressed as mean \pm SEM percentage of the mean control group. *indicates significant difference from control, $p < 0.05$; $n = 6/\text{group}$.

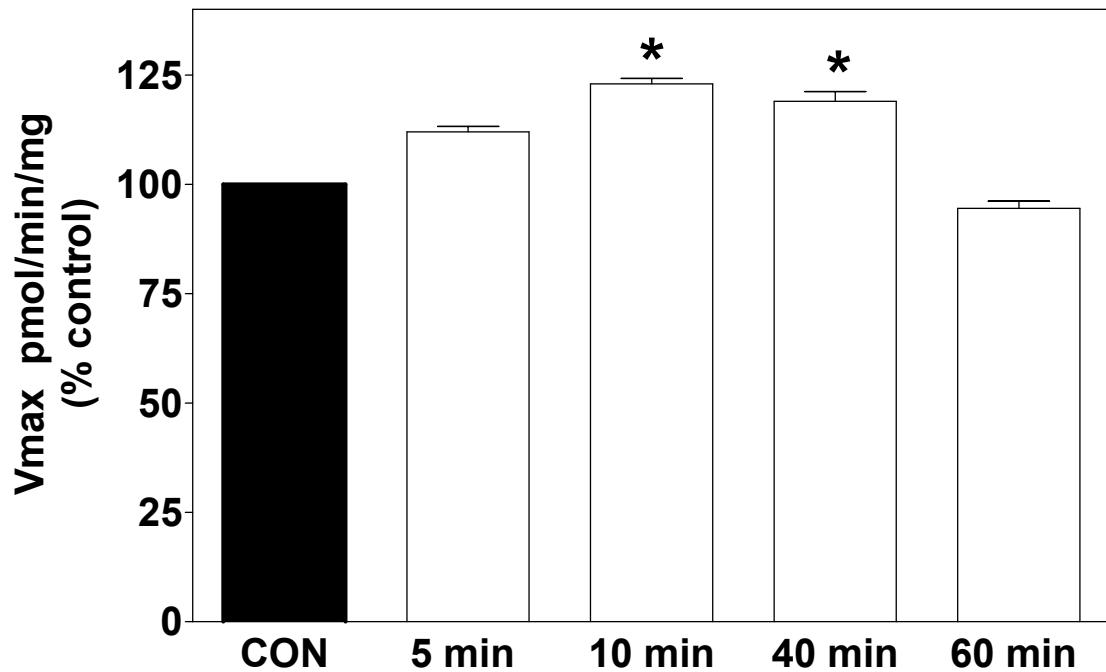


Table 2. Nicotine pretreatment does not alter the K_t in striatal synaptosomes measured by [^3H]DA uptake.

Time (min)	K_t (μM)	
	Saline	Nicotine (0.8 mg/kg)
5	0.09 ± 0.03	0.12 ± 0.03
10	0.09 ± 0.03	0.09 ± 0.03
40	0.08 ± 0.03	0.11 ± 0.03
60	0.10 ± 0.03	0.10 ± 0.02

Table 3. Nicotine (0.8 mg/kg, s.c.) pretreatment does not alter [³H]GBR 12935 binding to rat striatal membranes.

Time (min)	B _{max} (pmol/mg protein)		K _d (μM)	
	SALINE	NICOTINE	SALINE	NICOTINE
5	183 ± 22	192 ± 33	3.3 ± 0.4	4.6 ± 1.0
10	290 ± 35	195 ± 32	5.3 ± 1.0	4.4 ± 0.5
40	277 ± 69	229 ± 37	4.8 ± 1.0	5.0 ± 0.7

Data are expressed as Mean ± S.E.M, n = 4-9/group.

Figure 22. Biotinylation assays revealed no change in the distribution of striatal DAT at 10 or 40 min following nicotine administration.

Representative immunoblots (*Top panel*) for DAT, PP2A, calnexin and β -actin in total, biotinylated (BIOT) and nonbiotinylated (NB) fractions of striatal synaptosomes obtained 10 and 40 min following nicotine (0.32 mg/kg; s.c.) or saline injection. Group data for DAT in total, biotinylated (BIOT) and nonbiotinylated (NB) fractions of striatal synaptosomes obtained 10 and 40 min following nicotine or saline injection are presented as mean \pm S.E.M, n = 5/group (*Bottom panel*). Immunoreactive bands were within the linear range of detection. Biotinylation assays used β -actin as a control for protein loading. PP2A and calnexin were used to monitor biotinylation of proteins located in intracellular compartments and were found in the nonbiotinylated fractions.

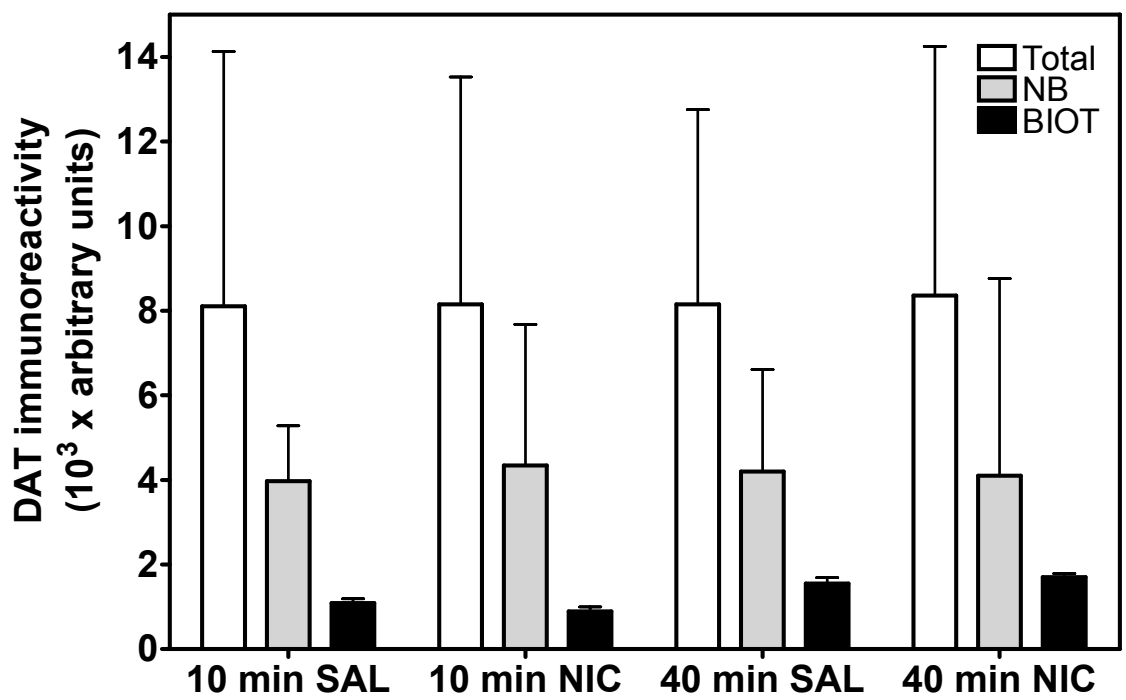
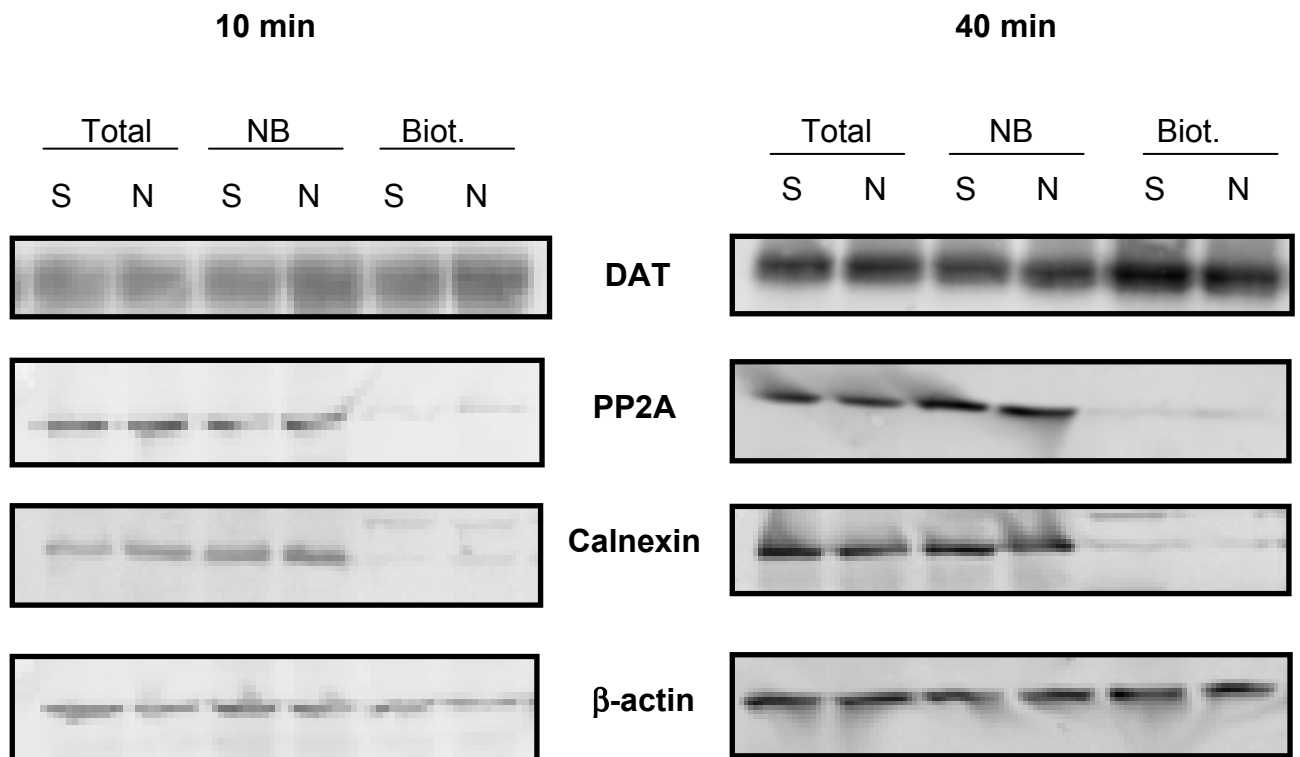
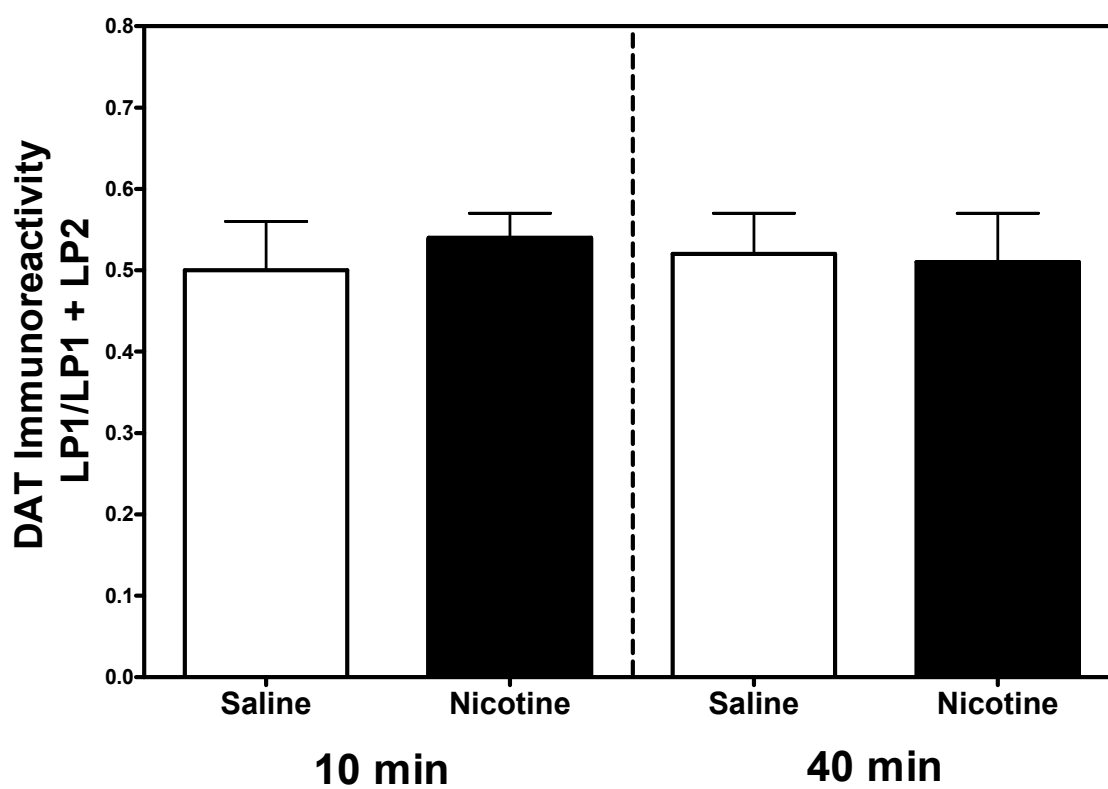
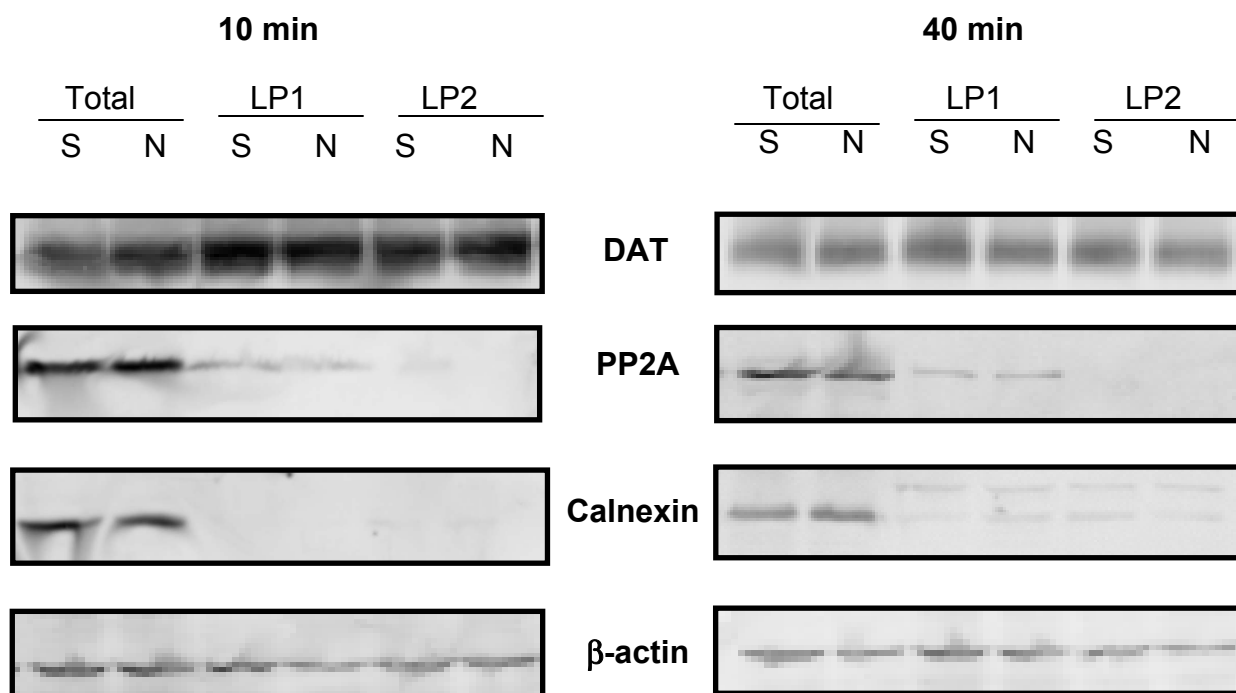


Figure 23. Subfractionation assays revealed no change in the distribution of striatal DAT at 10 and 40 min following nicotine administration.

Representative immunoblots of DAT, PP2A, calnexin and β -actin in total, LP1 (synaptic plasma membrane) and LP2 (vesicular) fractions of striatal synaptosomes from nicotine (0.32 mg/kg; s.c.) or saline injected rats 10 and 40 min post-injection (*Top panel*). Group data for DAT expressed as the LP1/LP1+LP2 ratio at 10 and 40 min following nicotine or saline injection (*Bottom panel*). LP1/LP1+LP2 ratio represent fraction of DAT in the plasma membrane fraction as ratio of DAT in plasma membrane and vesicular fraction. Data are presented as mean \pm S.E.M, n = 5/group. The levels of DAT-immunoreactivity ($\times 10^5$ arbitrary units) in total fractions were 18.0 ± 1.34 (saline, 10 min), 16.8 ± 2.37 (NIC, 10 min), 13.8 ± 1.36 (saline, 40 min) and 15.2 ± 1.92 (NIC, 40 min). Immunoreactive bands that were quantified were within the linear range of detection. β -actin was used as a protein loading control.



Chapter Six

Discussion and Conclusions

I. Review

Tobacco dependence is the most common drug of abuse and the leading preventable cause of death in the USA (Surgeon General's Report, 1988; Jaffe, 1990; USDHS, 2001). Nicotine, the most abundant alkaloid in tobacco (Bush et al., 1993), has intrinsic rewarding properties, which are believed to be responsible for tobacco dependence (Koob, 1992; Corrigall et al., 1992; Stolerman and Jarvis, 1995; Di Chiara, 2000; Balfour, 2002; Mathieu-Kia et al., 2002; Garrett et al., 2003). Volunteers who habitually smoke and are tobacco deprived will self-administer nicotine (Henningfield and Goldberg, 1983; Harvey et al., 2004; Johnson et al., 2004). Indicative of its reinforcing properties, nicotine is self-administered in animal models (Goldberg et al., 1981; Henningfield and Goldberg, 1983; Cox et al., 1984; Corrigall and Coen, 1989; Corrigall et al., 1992, 1994; Sannerud et al., 1994; Donny et al., 1996, 2003; Rose and Corrigall, 1997; Shoaib et al., 1997; Valentine et al., 1997; Rasmussen and Swedberg, 1998; Caggiula et al., 2001, 2002; Suto et al., 2001; LeSage et al., 2002, 2003; Fu et al., 2003; Paterson et al., 2004). While nicotine and acetylcholine both act at the nicotinic receptor, the brain responds differently to nicotine than acetylcholine. Acetylcholine undergoes rapid metabolism by acetylcholinesterase, whereas nicotine remains in the brain longer with a $t_{1/2}$ of 52 min (Ghosheh et al., 1999). Thus, the effects of nicotine on synaptic events are prolonged relative to acetylcholine. Therefore, the effect of nicotine on the DA system, and specifically

DAT which is responsible for regulating extracellular DA concentrations, is important in the overall function of the DA system.

Previous research on the neurobiology of reward and drug addiction has focused on the mesocorticolimbic and nigrostriatal DA systems, emphasizing the role of the nucleus accumbens, MPFC and striatum. Recently, focus has been placed on the involvement of the striatum and MPFC in reward and reinforcement. The MPFC, which is innervated by dopaminergic projections from the ventral tegmental area, is believed to encode secondary conditioned stimuli associated with environmental cues paired with drug, leading to reward expectancy, which is recognized as important to the process of addiction and relapse to drug use (Berridge and Robinson, 1998; Shima and Tanji, 1998; Kelley, 1999; DiChiara, 2000; Kelley and Berridge, 2002; Peoples, 2002; Cohen et al., 2004; DiChiara et al., 2004; Rose and Behm, 2004; Brody et al., 2004a). Integration of the motivational information from the MPFC occurs at least in part in striatal neurons, which are innervated by dopaminergic projections from the substantia nigra, leading to the initiation and execution of movement in reward expectancy and detection of reward (Martin-Soelch et al., 2001).

The experiments conducted in this dissertation project have shown that nicotine administration (s.c.) increases DA clearance in rat striatum and MPFC and this effect is inhibited by mecamylamine, suggesting nicotinic receptor involvement. Furthermore, tolerance develops to the nicotine-induced increase

in DA clearance following repeated nicotine administration in both striatum and MPFC. The nicotine-induced increase in DA clearance in striatum is due to an increase in the Vmax of DAT without an increase in trafficking of DAT to the cell surface. Finally, the nicotine metabolite and tobacco alkaloid, nornicotine, was shown to have an opposite action of nicotine, i.e., nornicotine administration decreases DA clearance in striatum. The current studies are the first to examine the effect of nicotine on DAT function in vivo in both the striatum and MPFC. Furthermore, these are the first studies examining the mechanism by which nicotine modulates DAT function.

II. Nicotinic receptor modulation of DAT function in striatum and MPFC

Using in vivo voltammetry, systemic nicotine, in a dose-related manner, increases DA clearance in both striatum and MPFC. In both brain regions, nicotine increased DA clearance by 50%. However, across the same nicotine dose range, differential patterns in the nicotine dose-response curve were observed in striatum and MPFC. That is, a monophasic dose-response curve was observed in striatum, whereas a U-shaped curve was found in MPFC. Maximal effect occurred at a lower dose in MPFC than in striatum (0.4 and 0.8 mg/kg, respectively). With respect to the time course of the nicotine effect, in both brain regions, the onset of a significant effect on DA clearance occurred 10 to 15 min after nicotine injection. The time to maximal response was more rapid in MPFC compared with striatum (30 and 45 min, respectively). The time course of the response to nicotine in both brain regions is in good agreement with

pharmacokinetic data showing a maximal nicotine concentration in rat brain at 5 min after peripheral nicotine injection and a brain $t_{1/2}$ of 52 min (Ghosheh et al., 1999). Nevertheless, the pattern of the nicotine dose-response curve in the present study was different between MPFC and striatum, with MPFC showing greater sensitivity to nicotine. α -Conotoxin MII inhibits nicotine-evoked [3 H]DA overflow by only 50%, implicating involvement of at least two different nicotinic receptor subtypes (Zoli et al., 2002; Salminen et al., 2004; Azam and McIntosh 2005). Nicotine does not evoke DA release in microdialysis experiments using α 4-knockout mice (Marubio et al., 2003), indicating that α 4 nicotinic receptors are critical for DA release, at least in ventral striatum. Previously, it was reported that nicotine was able to enhance amphetamine-evoked [3 H]DA release from rat MPFC slices, but not from striatal or nucleus accumbens slices *via* an action at nicotinic receptors (Drew et al., 2000). Thus, it is possible that striatal nicotinic receptors modulating DAT function are different between those in MPFC.

The present study also demonstrates that the nicotine-induced enhancement of DA clearance in both MPFC and striatum was inhibited by pretreatment of the rats with mecamylamine, a nonselective nicotinic receptor antagonist (Varanda et al., 1985). The hallmark of an action at nicotinic receptors is its blockade by mecamylamine. Thus, the current study utilized mecamylamine as the first approach to determine nicotinic receptor involvement in the nicotine-induced increase in DA clearance. Further studies are warranted examining the effects of subtype selective antagonists to determine if different

subtypes are involved in modulating DAT function between striatum and MPFC. These results suggest that nicotine stimulates nicotinic receptors to increase DAT function in these two brain regions. The current results are in agreement with previous studies showing that peripheral nicotine administration increased DA clearance in the nucleus accumbens (Hart and Ksir, 1996). In the current study, mecamylamine had no effect on its own, but inhibited the effect of nicotine on DA clearance in both striatum and MPFC. While mecamylamine has been shown to act at NMDA receptors as well as nicotinic receptors (O'Dell and Christensen, 1988), the hallmark of an action at nicotinic receptors is blockade by mecamylamine. Thus, mecamylamine was used in the current studies to determine involvement of nicotinic receptors on the nicotine-induced increase in DA clearance. Hart and Ksir (1996) also reported no effect of mecamylamine alone on DAT function in nucleus accumbens. It is possible that the nicotinic receptors modulating DA release and DAT function are different. Since acetylcholine is the endogenous ligand for the nicotinic receptor, it is likely that there is some basal activity of DA release based on nicotinic receptor activation by acetylcholine. The observation that mecamylamine had no effect alone suggests that nicotinic receptors, which modulate DAT function in these brain regions, are not tonically activated. These results support the first hypothesis that nicotine will increase DA clearance in the striatum and MPFC in a dose-dependent manner, and this increase in clearance will be mediated by nicotinic receptors.

Following nicotine-induced DA release DA can be taken back up into the terminal by DAT and can also stimulate presynaptic DA D2 autoreceptors. D2 autoreceptor activation decreases both DA synthesis and DA release from presynaptic terminals (Starke et al., 1989; Langer, 1997). Importantly stimulation of D2 autoreceptors also modulates DAT function. Uptake of DA into striatal minces and synaptosomes is increased following application of a D2 receptor agonist, quinpirole (Meiergerd et al., 1993; Batchelor and Schenk, 1998; Thompson et al., 2001). Furthermore, the quinpirole-induced increase in DA uptake is inhibited by spiperone, a D2 receptor antagonist (Thompson et al., 2001). Local application of the D2 receptor antagonist raclopride decreased DA clearance in striatum, nucleus accumbens and prefrontal cortex (Cass and Gerhardt, 1994). In D2 receptor knockout mice, DA clearance is decreased by 50% compared to wild-type mice (Dickinson et al., 1999). It is possible that the nicotine-induced increase in DA clearance observed in the current studies may be influenced by nicotine-induced DA release followed by activation of D2 receptors, as stimulation of D2 receptors would lead to an increase in DA clearance.

III. Mechanisms Underlying the Different Shaped Dose-Response Curves observed in Striatum and MPFC.

IIIa. Different nicotinic receptors modulating DA clearance in striatum and MPFC.

Heteromeric $\alpha 4\beta 2^*$ nicotinic receptors are most abundant in brain, and second most abundant are homomeric $\alpha 7^*$ nicotinic receptors (Whiting and Lindstrom, 1986; Wada et al., 1989; Morris et al., 1990; Schoepfer et al., 1990; Anand et al., 1991; Flores et al., 1992; Lukas et al., 1999). The subunits display different, but overlapping, patterns of expression in brain (e.g., $\beta 2$ mRNA expression is prominent in the cerebellum, which exhibits no $\alpha 4$ hybridization; Wada et al., 1989). mRNA for nine subunits ($\alpha 2$ – $\alpha 7$ and $\beta 2$ – $\beta 4$) of nicotinic receptors have been identified in SN and VTA dopaminergic neurons (Klink et al., 2001, Zoli et al., 2002; Azam et al., 2002; Wooltorton et al., 2003), indicating that potentially a large number of heteromeric nicotinic receptor subtypes of pentameric structure may be expressed by these neurons.

Nicotinic receptors are localized on presynaptic terminals and cell bodies of VTA and SN DA neurons (Schwartz et al., 1984; Clarke and Pert, 1985; McGehee and Role, 1995; Wonnacott, 1997; Quik et al., 2005); and evidence indicates that nicotinic receptor subtypes at cell body and terminal locations differ pharmacologically (Reuben et al., 2000; Champtiaux et al., 2003). In striatal synaptosomes, nicotine has a lower affinity for nicotinic receptors than acetylcholine, and acetylcholine has equal affinity to epibatidine (a nicotinic receptor agonist; Reuben et al., 2000). However, in a dendrosomal preparation, nicotine has equal affinity to epibatidine (Reuben et al., 2000), suggesting that the nicotinic receptors in the terminal regions differ from those in the cell body regions. Furthermore, $\alpha 6\beta 2^*$ receptors have been shown to be located on DA

terminals, whereas non $\alpha 6(\alpha 4\beta 2)^*$ receptors represent the majority of functional receptors on DA cell bodies (Champtiaux et al., 2003; Salminen et al., 2004). Importantly, α -conotoxin MII inhibits nicotine-evoked [^3H]DA overflow by only 50%, implicating involvement of at least two different nicotinic receptor subtypes (Zoli et al., 2002; Salminen et al., 2004; Azam and McIntosh 2005). It is likely that the difference in pharmacology between the nicotinic receptors in the cell body regions and the nerve terminal regions play different physiological roles.

The involvement of distinct nicotinic receptor subtypes in striatum and MPFC provides a likely explanation for the differential nicotine dose-response pattern observed in the current study. Recent studies have demonstrated the presence of multiple mRNAs for nicotinic receptor subunits ($\alpha 2$ - $\alpha 7$ and $\beta 2$ - $\beta 4$) and their respective proteins in DA cell bodies in both substantia nigra and ventral tegmental area; however, differences in the relative abundance of these subunits in substantia nigra and ventral tegmental area have also been reported (Klink et al., 2001; Azam et al., 2002; Zoli et al., 2002; Wooltorton et al., 2003). The nicotinic receptors expressed in the nigrostriatal and mesocorticolimbic DA systems depend on the specific combinations of subunits forming functional nicotinic receptors. In striatum, the primary subtypes thought to be expressed are the $\alpha 6\beta 2^*$ and $\alpha 4(\text{non } \alpha 6)\beta 2^*$ nicotinic receptors (Zoli et al., 2002). Pairwise expression of nicotinic receptor subunits in *Xenopus* oocytes initially revealed characteristic pharmacological profiles, i.e., relative sensitivity and responsiveness to a range of nicotinic ligands (Luetje and Patrick, 1991).

Inclusion of a third type of subunit (e.g., $\alpha 5$ with $\beta 3$ and $\beta 2$ subunits) in similar expression systems further altered the physiological response, calcium permeability, and desensitization characteristics of the expressed nicotinic receptor subtypes (Gerzanich et al., 1998). Characterization of the physiological response of individual neurons in midbrain slices revealed four different patterns of nicotinic receptor-mediated currents, revealing the complexity of native nicotinic receptors, which purportedly contained as many as four different subunits (Klink et al., 2001). More recently, the selective $\alpha 6$ nicotinic receptor antagonist, α -conotoxin MII, has been shown to decrease DA release, suggesting the involvement of the $\alpha 6$ subunit in nicotine-evoked DA release (Salminen et al., 2004). Importantly, α -conotoxin MII inhibits nicotine-evoked [^3H]DA overflow by only 50%, implicating involvement of at least two different nicotinic receptor subtypes (Zoli et al., 2002; Salminen et al., 2004; Azam and McIntosh 2005). Immunoprecipitation of nicotinic receptors from DA terminals as well as electrophysiological studies in DA neurons from various nicotinic receptor knockout mice suggest the involvement of $\alpha 4\beta 2^*$, $\alpha 6\beta 2^*$, $\alpha 6\alpha 4\beta 2^*$ nicotinic receptors in DA release (Champtiaux et al., 2003).

IIIb. Neuronal circuitry is different between striatum and MPFC.

Another potential explanation for the difference in regional dose-response is that the local neuronal circuitry is different between these two brain regions, i.e., different afferents impinge on the dopaminergic terminals in striatum and

MPFC potentially providing differential regulation of DAT function. In this regard, the U-shaped function in MPFC may be the result of nicotine-induced stimulation of an additional neurotransmitter system in the MPFC. The result of activation of the additional neurotransmitter may have opposed the nicotinic receptor-mediated enhancement of DAT function. For example, high concentrations of nicotine have been shown to activate $\alpha 7$ nicotinic receptors, resulting in glutamate release in the frontal cortex (Schilstrom et al., 2000; Marchi et al., 2002). Stimulation of metabotropic glutamate receptors has been reported to decrease DAT function (Page et al., 2001). Thus, activation of $\alpha 7$ receptors indirectly through glutamate neurotransmission could result in the inhibition of DAT function, counteracting activation of the high affinity heteromeric nicotinic receptors, which enhance DAT function.

Alternatively, nicotinic receptors may modulate DAT function through activation of neural circuitry at the cell body level. Local administration of nicotine into the substantia nigra and ventral tegmental area has been shown to evoke DA release in striatum and nucleus accumbens, respectively, via stimulation of nicotinic receptors in the cell body region (Blaha and Winn, 1993; Sziraki et al., 2002). Additionally, the effect of peripheral administration of nicotine to increase DA release in the nucleus accumbens determined using microdialysis was inhibited by local administration of mecamylamine into the ventral tegmental area (Sziraki et al., 2002). Thus, it seems plausible that in the current study, peripherally administered nicotine may be acting at nicotinic receptors at the level

of the cell body to modulate DAT function at the terminal. Furthermore, different nicotinic receptor subtypes expressed at the cell body may be responsible for the different dose-response patterns observed in MPFC and striatum with respect to DA clearance.

Due to the lower DAT density and decreased number of DAT per terminal in MPFC compared with striatum, metabolism and diffusion likely play a larger role in clearing DA from the extracellular space in MPFC under physiological conditions. However, following pharmacological treatment with nicotine, enhanced DAT function in MPFC would be predicted to have a large impact on dopaminergic transmission. Nicotine enhancement of DAT function would result in more efficient DA clearance from the extracellular space.

IV. Data Analysis

Initially the voltammetry data was presented and analyzed as described in the literature. The results of the analysis were problematic and confusing because the clearance data were showing a decrease in clearance when actually the results showed an increase in clearance (Figure 8). Further examination of the data presented in the literature revealed that previous investigators were not using a correct or most appropriate measure of clearance. Exogenous DA clearance has been defined previously as A_{\max}/T_{80} , a parameter with units of concentration/time (Zahniser et al., 1999). In more recent assessments of DAT

function by this group of investigators (Sabeti et al., 2002), another parameter for DA clearance has been elaborated and referred to as DA clearance efficiency. DA clearance efficiency has been defined as a rate constant k , with units of sec^{-1} and is determined by fitting the DA signal to a first-order exponential decay function ($A(t)=A_{\text{max}}*e^{-k(t-t_0)}$); where A is the signal amplitude (μM) at any time t (sec) following peak signal amplitude (A_{max}), and k is the first-order rate constant of decay of the DA signal). However, exogenous DA clearance defined by either A_{max}/T_{80} or k do not represent the classical physiological measure of clearance. In the current studies, the classical pharmacokinetic approach has been utilized to assess exogenous DA clearance (Ito et al., 1998; Shargel et al., 2005). Thus, the parameter determined in the current study, CL_{DA} , was calculated as the amount of DA pressure ejected divided by the AUC for each DA signal, with units of volume/time. CL_{DA} can be directly equated to the physiological process of DA elimination from the ejection site, and represents DA transport via DAT. Importantly, CL_{DA} is independent of the concentration of DA that is pressure ejected and is independent of the volume into which DA distributes under linear conditions. The current work used a more classical approach for the analysis of the clearance parameter, which allowed for a more accurate measurement of DA clearance. Thus, the current analysis directly measures the efficiency of DA removal after its exogenous application, i.e., the transport process via DAT.

V. Tolerance Develops to the nicotine-induced increase in DAT function following repeated nicotine administration.

Following repeated nicotine administration tolerance develops to the nicotine-induced increase in DAT function in MPFC and striatum. Following repeated injection with saline, an acute challenge with nicotine resulted in an increase in DA clearance in MPFC and striatum consistent with the findings presented in Chapter Two (Middleton et al., 2004). Moreover, following repeated treatment with nicotine, DA clearance after nicotine challenge was not different from that in the control group repeatedly administered saline and challenged with saline. However, in both MPFC and striatum, DA clearance in the group repeatedly injected with nicotine and challenged with nicotine on the experiment day was decreased compared to the group of rats injected repeatedly with saline and challenged with nicotine on the experiment day. These results demonstrate that tolerance develops to the nicotine-induced increase in DA clearance following repeated nicotine injection. The current results extend previous findings showing that systemically administered nicotine increases DA clearance, *via* a nicotinic receptor-mediated mechanism, in several dopaminergic terminal regions, including MPFC, striatum, and nucleus accumbens (Hart and Ksir, 1996; Middleton et al., 2004). The current results did not support the third hypothesis that repeated nicotine administration will further enhance the nicotine-induced increase in DA clearance in striatum and MPFC.

Va. Acute vs. Repeated Nicotine Administration

It is possible that initially nicotinic receptor activation causes release of DA and subsequently increases the clearance of DA from the synapse. The

increase in DA clearance may cause a sharpening of the DA kinetics. Following nicotine pretreatment, using *in vitro* superfusion and *in vivo* microdialysis, nicotine-evoked DA release was higher than nicotine-evoked DA release from rats injected with saline (Yu and Wecker, 1994; Marshall et al., 1997). A study examining the chronic use of tobacco in humans determined that DA levels were elevated in the striatum of smokers compared to nonsmokers (Court et al., 1998), which would lead to an increase in DA activity. Acutely, the increase in DA clearance coupled with the immediate increase in DA release may sharpen the kinetics of the nicotine-mediated increase in extracellular DA concentration. By sharpening the kinetics, the extracellular DA is removed from the synapse more rapidly. In the current study, tolerance that occurs following repeated nicotine administration may cause a flattening of the DA kinetics, thus increasing the amount of DA in the extracellular space.

Tolerance to chronic nicotine administration has been shown in various animal models, i.e., locomotor activity, striatal dopamine metabolism and of nicotine-induced antinociception (Stolerman et al., 1973; Marks et al., 1983; Marks et al., 1991; McCallum et al., 2000; Pietila and Ahtee, 2000). Another potential explanation for the tolerance of DA clearance to repeated nicotine administration may be due to nicotinic receptor desensitization. Following long-term stimulation of nicotinic receptor by agonists, nicotinic receptors are no longer able to produce an effect, even in the presence of agonist, i.e. desensitization. Furthermore, the EC₅₀ for nicotine to produce desensitization

was the same as that required for upregulation, suggesting that upregulation occurs as a result of desensitization (Fenster et al., 1999). Using various doses of nicotine and different dosing regimens, Rowell and Li (1997) showed that nicotinic receptors were upregulated and that the degree of desensitization was dependent on dose and regimen of administration. Concentrations of nicotine that are achieved by smokers desensitize nicotinic receptors located on the mesolimbic DA neurons (Pidoplichko et al., 1997). Tolerance following chronic nicotine has been shown in the absence of upregulation of nicotinic receptors (Pauly et al., 1992; McCallum et al., 2000). However, it is also possible that the observed tolerance is due to nicotinic receptor upregulation. Mice chronically treated with nicotine have shown an increase in nicotine binding sites in brain (Marks et al., 1983; Schwartz and Kellar 1983; Collins et al., 1988; Kassiou et al., 2001; Nuutinen et al., 2005). Recently, in $\beta 2$ knockout mice, following chronic nicotine treatment, upregulation of nicotine binding sites is no longer observed (McCallum et al., 2005). Thus, while acute nicotine increases DAT function, repeated nicotine decreases DAT function back to control levels.

Tolerance to the nicotine-induced increase in DA clearance may also be due to desensitization of DA D2 autoreceptors. Following repeated nicotine injection, dopamine release is enhanced compared to acute nicotine injection (Yu and Wecker, 1994; Marshall et al., 1997). As mentioned above, D2 receptors can modulate DAT function. Thus, it is possible that following repeated nicotine injection the increased amount of extracellular DA results in desensitization of D2

autoreceptors and therefore DAT function is no longer increased. This would then result in tolerance to the nicotine-induced increase in DAT function.

Previously, it was reported that nicotine was able to enhance amphetamine-evoked [³H]DA release from rat MPFC slices, but not from striatal or nucleus accumbens slices *via* an action at nicotinic receptors (Drew et al., 2000). More recently, the same group examined the effect of chronic nicotine on amphetamine evoked DA release (Drew and Werling, 2003). Following chronic nicotine treatment, nicotine was still able to enhance amphetamine-stimulated DA release, suggesting that even after chronic nicotine; nicotinic receptors may not be desensitized. In contrast, the current studies determined that following repeated nicotine administration, tolerance develops to the nicotine-induced increase in DA clearance. It is possible that the nicotinic receptors modulating DA release and DA transport are different. Thus, one subtype may desensitize (nicotine can still release DA) and one may not (nicotine can no longer modulate DAT function).

Tolerance to the nicotine-induced increase in DA clearance may be due to an increase in nornicotine production. Nornicotine decreases DAT function (Chapter Three). Following systemic nicotine injection, nicotine reaches maximal brain concentration within 5 min and has a brain $t_{1/2}$ of 52 min. However, nicotine metabolism results in production of nornicotine (Ghosheh et al., 1999). Following

nicotine injection nornicotine reaches maximal brain concentrations 60 min following nicotine injection and has a brain $t_{1/2}$ of 166 min (Ghosheh et al., 1999). Furthermore, following repeated nicotine treatment, nornicotine accumulates in brain (Ghosheh et al., 2001). Therefore, it is possible the nicotine is no longer able to increase DAT function following repeated nicotine treatment because nornicotine is inhibiting DAT function. Following repeated nicotine administration it is possible that changes in nicotine metabolism also may occur. Cytochrome P450 (CYP) 2B1 is a nicotine metabolizing enzyme found in the brain. Following 7 days of nicotine treatment, CYP 2B1 protein was increased in brain, specifically in the brain stem, frontal cortex, striatum and olfactory tubercle (Miksys et al., 2000). Increases in the CYP 2B1 protein could lead to an increase in nicotine metabolism. Thus, the difference observed between acute and repeated nicotine in striatum and MPFC may be due to differences in nicotine metabolism.

VI. Nornicotine inhibits DA clearance.

Attention has focused on the *N*-demethylated nicotine metabolite and minor tobacco alkaloid, nornicotine, as contributing to the neuropharmacological effects of nicotine exposure and tobacco use (Crooks and Dwoskin, 1997). Nornicotine inhibits [3 H]nicotine binding to rat brain membranes with a 50-fold lower affinity compared with nicotine (Reavill et al., 1988; Copeland et al., 1991; Zhang and Nordberg, 1993; Xu et al., 2001). In contrast, nicotine and nornicotine exhibit similar affinities for the [3 H]methyllycaconitine binding site in brain. These results indicate interaction of these alkaloids with both $\alpha 4\beta 2^*$ and $\alpha 7^*$ nicotinic

receptors. Similar to nicotine, nornicotine evokes a concentration-dependent, Ca^{2+} -dependent and mecamylamine-sensitive increase in DA release from rat striatal and nucleus accumbens slices (Dwoskin et al., 1993; Teng et al., 1997; Green et al., 2001), indicating that nornicotine acts as an agonist at nicotinic receptor subtypes modulating DA release. In the latter studies, whereas nicotine and nornicotine were equipotent in releasing DA from striatal slices, nicotine was more potent than nornicotine in releasing DA from nucleus accumbens slices. Furthermore, nornicotine is a partial agonist whereas nicotine is a full agonist (Green et al., 2001). It is interesting to note that nornicotine has a longer half-life than nicotine in plasma and brain (Kyerematen et al., 1990; Crooks et al., 1995, 1997; Ghosheh et al., 2001); and following chronic treatment with nicotine, nornicotine accumulates in brain reaching pharmacologically relevant concentrations (Ghosheh et al., 2001). The current experiments examined the ability of nornicotine to modulate striatal DAT function. Using in vivo voltammetry, systemic administration of nornicotine decreased DA clearance in striatum in a dose-related manner, demonstrating that nornicotine inhibits striatal DAT function. The nornicotine-induced decrease in DA clearance was inhibited completely by mecamylamine, a noncompetitive and nonselective nicotinic receptor antagonist, indicating that this effect of nornicotine on DAT function is mediated by nicotinic receptors. In contrast, nornicotine did not inhibit [^3H]DA uptake into striatal synaptosomes when nornicotine was incubated with synaptosomes obtained from drug-naïve rats. Similar to nornicotine, incubation of synaptosomes from drug-naïve rats with nicotine did not inhibit [^3H]DA uptake.

Taken together, these results suggest that nornicotine and nicotine activate nicotinic receptors which are located at sites other than on DA nerve terminals in striatum to inhibit DAT function *in vivo*. The current results did not support the hypothesis that nornicotine would increase DAT function in striatum, however, the effect of nornicotine was mediated by nicotinic receptors.

VII. Potential Mechanism for the Differences Observed between Nicotine and Nornicotine.

VIIa. Nicotinic receptor subtypes.

More than one nicotinic receptor subtype has been suggested to mediate nicotine-evoked DA release in striatum (Kaiser et al., 1998; Dwoskin et al., 2004). α -Conotoxin MII as well as other small molecules is a nicotinic receptor antagonist that partially inhibits DA release, i.e., only inhibits DA release by 50% (Wilkins et al., 2002; Grinevich et al., 2003; Wei et al., 2005). This has led to the idea that there are more than one nicotinic receptor subtype that modulate DA release, α -conotoxin MII sensitive and α -conotoxin MII resistant subtypes (Luetje, 2004). A recent study examined the subunit composition of nicotinic receptors modulating α -conotoxin MII sensitive and resistant DA release in striatal synaptosomes and slices using knockout mice for various nicotinic receptor subunits (Salminen et al., 2004). Both the α -conotoxin MII sensitive and resistant subtypes required the β 2 subunit, however only the α -conotoxin MII sensitive subtype required the β 3; whereas the α -conotoxin MII resistant subtypes required α 4. These data suggest that the primary subtypes involved in

DA release are the $\alpha 6\beta 3\beta 2^*$ and $\alpha 4\beta 2^*$ subtypes (Salminen et al., 2004). High concentrations of nicotine have been shown to activate $\alpha 7^*$ nicotinic receptors, which results in glutamate release in the frontal cortex (Schilstrom et al., 2000; Marchi et al., 2002). Stimulation of metabotropic glutamate receptors has been reported to decrease DAT function (Page et al., 2001). Thus, multiple nicotinic receptor subtypes could be involved in modulation of DAT function. The current evidence provides the first report that more than one nicotinic receptor subtype may mediate DAT function, since nicotine and nornicotine both mediate DAT function but in a different manner.

VIIb. Neuronal Circuitry

The current study also shows that incubation of synaptosomes from drug-naïve rats with both nornicotine and nicotine does not alter [^3H]DA uptake into striatal synaptosomes, which is consistent with previous reports determining the effect of nicotine in vitro (Carr et al., 1989; Zhu et al., 2003). Taken together with the results from the in vivo voltammetry studies, these findings suggest that the nicotinic receptors mediating DAT function in vivo are located on dopaminergic cell bodies in the substantia nigra or in brain regions other than striatum. However, procedural differences between the in vivo and in vitro preparations, such as the use of the urethane anesthetic in vivo, may also contribute to the differential results obtained. The anesthetic used in the current in vivo voltammetry experiments, urethane, has been shown to not alter striatal DAT function (Sabeti et al., 2003).

Evidence supporting a critical role for nicotinic receptors at the level of the dopaminergic cell body is provided from studies in which local administration of nicotine into the substantia nigra or ventral tegmental area evokes DA release in striatum and nucleus accumbens, respectively (Blaha and Winn, 1993; Nisell et al., 1994; Sziraki et al., 2002). Additionally, local administration of mecamylamine into the ventral tegmental area was shown to inhibit DA release in the nucleus accumbens following peripheral administration of nicotine (Sziraki et al., 2002). Thus, as has been suggested for nicotine, nicotinic receptors at the level of the substantia nigra may be involved in modulating DAT function in striatum following peripheral nicotine administration.

VIII. Trafficking Independent modulation of DAT function.

The current project as well as others have examined the effect of nicotine on DA uptake/transport by exposing synaptosomes to nicotine in vitro (Carr et al., 1989; Zhu et al., 2003). The results of those studies showed that nicotine did not alter DA transport. In the current studies the effect of nicotine administered in vivo on DAT function in vitro were examined. Furthermore, previous studies examined the ability to inhibit DAT function. In the current study, a saturation analysis was performed to determine the kinetic parameters of in vivo nicotine administration on DAT function measured in vitro. In rats pretreated with nicotine (0.8 mg/kg, s.c.) and killed 10 and 40 min later, a significant 20-25% increase in the V_{max} for [3H]DA uptake was observed in striatal synaptosomes. The dose of

nicotine was chosen based on previous results showing that nicotine (0.8 mg/kg) increased DA clearance in vivo in rats striatum. Rats killed at 5 min following nicotine injection showed a slight but nonsignificant increase in [³H]DA uptake. This effect was diminished to control levels by 60 min following nicotine injection. This increase in DA uptake was not accompanied by an increase in the total amount of DAT protein as measured by [³H]GBR 12935 binding. Furthermore, nicotine pretreatment did not increase the density of DAT on the cell surface membrane as measured using biotinylation and subcellular fractionation experiments. These data support and extend previous findings showing that systemic nicotine increases DA clearance in vivo in rat striatum (Middleton et al., 2004). The increase in DA clearance in striatum observed in vivo is likely due to an increase in the V_{\max} of DAT. Taken together, these data suggest that nicotine increases DA uptake in striatum in vitro through a trafficking-independent mechanism. These data support the hypothesis that nicotine increases DAT function by increasing the V_{\max} for DA uptake, however, the results did not validate the hypothesis that the nicotine-induced increase in DAT function was due to an increase in the number of transporters on the cell surface.

VIIIa. DAT Modulation by different proteins/phosphorylation

DAT localized at the presynaptic membrane reflects functional DAT that is involved in the clearance of extracellular DA. The mechanism by which nicotine modulates DAT function may involve regulation of intracellular signaling cascades. Changes in DAT surface expression have been shown to be induced

by activation of protein kinase C or exposure to psychostimulants, and such changes correlate with alterations in DA uptake in striatum (Copeland et al. 1996; Chi and Reith, 2003; Vaughan et al., 1997) and in cell systems expressing DAT (Pristupa et al. 1998). In cells expressing hDAT, application of amphetamine results in a decrease in the cell surface expression of DAT (Kahlig et al., 2004). Whereas amphetamine decreases cell surface DAT, cocaine has been shown to increase trafficking of DAT to the cell surface (Little et al., 2002).

In the current study, nicotine increases DAT function without a change in the cell surface localization of DAT, thus acting differently than other psychostimulants. Nitric oxide may play a role in the nicotine-induced increase in DAT function. Nicotine releases nitric oxide from rat hippocampal slices (Smith et al., 1998). Nicotine also activates voltage gated Ca^{++} channels which increase intracellular Ca^{++} levels and can lead to an increase in the production of reactive oxygen species. Recently, L-arginine has been shown to increase DAT activity using rotating disk electrode voltammetry in striatum by a nitric oxide synthase pathway (Volz and Schenk, 2004). Nicotine activates many different second messenger pathways that may be involved in modulating DAT. For example, nicotinic receptor activation induces ERK phosphorylation mediated by CaM kinase and MAP kinase in PC12 cells (Nakayama et al., 2001). ERK phosphorylation then induces CREB phosphorylation. In hippocampal neurons, nicotine has been shown to activate the transcription factor CREB which is dependent on calmodulin and MAP kinase (Zhang et al., 2001; Hu et al., 2002).

PI-3 kinase and p38 MAP kinase activate a trafficking-independent pathway for NET regulation (Apparsundaram et al., 2001), suggesting that neurons likely have multiple pathways to modulate NE clearance capacity intrinsically. Similar to insulin regulation of NET, p38 MAP kinase has been shown to have similar effects on the serotonin transporter, i.e., an increase in serotonin uptake without a change in trafficking of the serotonin transporter (Zhu et al., 2005). A schematic representation of the multiple pathways that may be involved in nicotinic receptor modulation of DAT function is shown in Figure 24. Taken together, these results suggest the possibility that nicotine activates neural systems in the striatum that subsequently augment the activity of signaling cascades leading to an increase in DAT function without altering cell surface localization.

One potential explanation could involve the activation of second messenger signaling cascades. A study in hippocampal neurons revealed that nicotinic receptor activation resulted in an increase in intracellular calcium (Hu et al., 2002). The calcium then activated MAP kinase and calcium/calmodulin-dependent protein kinase pathways to activate the cyclic AMP response element binding protein producing transcriptional effects (Hu et al., 2002). Furthermore, inhibition of the MAP kinase pathway results in downregulation of DAT from the plasma membrane to intracellular pools as measured by biotinylation in human embryonic kidney 293 cells (Morón et al., 2003). Taken together, these data suggest a potential role of the MAP kinase pathway to increase DAT levels on

the plasma membrane, and thus, increasing DA clearance following acute administration of nicotine. Repeated nicotine may result in an inhibition of the MAP kinase pathway resulting in down regulation of DAT, and therefore, a loss in the nicotine-induced increase in DA clearance. Furthermore, it is possible that nicotinic receptor activation could lead to phosphorylation of DAT, which would decrease DAT function (Copeland et al., 1996; Vrindavanam et al., 1996; Huff et al., 1997; Vaughan et al., 1997; Melikian and Buckley, 1999; Chang et al., 2001). Therefore, changes in DAT function can be regulated not only by trafficking but by modulating the phosphorylation status of DAT.

IX. Implications:

Nicotinic receptor agonists have been shown to increase learning and memory in both rats and humans (Levin, 1992; Warburton, 1992; Levin and Simon, 1998; Levin et al., 1999; Ernst et al., 2001; Houlihan et al., 2001; Uzun et al., 2004). Due to the lower DAT density and decreased number of DAT per terminal in MPFC compared with striatum, metabolism and diffusion likely play a larger role in clearing DA from the extracellular space in MPFC under physiological conditions. However, following pharmacological treatment with nicotine, enhanced DAT function in MPFC would be predicted to have a large impact on dopaminergic transmission. Nicotine enhancement of DAT function would result in more efficient DA clearance from the extracellular space, and cortical function would be disinhibited. Thus, the ability of nicotinic receptors to modulate DAT function, and thereby extracellular DA concentration, may have

physiological importance with respect to nicotine enhancement of cognitive processes such as attention, learning, and memory, as well as important clinical relevance with respect to schizophrenia and drug abuse.

Nicotine is an effective pharmacotherapy for the cessation of tobacco smoking when used alone or in combination with other drugs or clinical programs (Po, 1993; Rose et al., 1994; Balfour and Fagerstrom, 1996; Glover and Glover, 2001; Karnath, 2002). However, despite efficacy with nicotine replacement, the majority of smokers continue to relapse, suggesting that more efficacious therapeutic agents are needed. Nornicotine may offer a beneficial alternative to nicotine as a tobacco use cessation agent. The pharmacokinetic profile of nornicotine, with its longer half-life and slower clearance compared to nicotine (Kyerematen et al., 1990; Crooks and Dwoskin, 1997; Ghosheh et al., 1999), may afford additional advantages over nicotine. Furthermore, since nornicotine is significantly less potent than nicotine in increasing blood pressure and heart rate (Mattila, 1963; Risner et al., 1988; Stairs et al., submitted 2005), the safety index for nornicotine may be greater than that for nicotine, especially among smokers with advanced cardiovascular disease.

The ability of nornicotine to decrease DAT function in vivo, as shown in the present study, may afford another advantage over nicotine as a tobacco use cessation agent. In this respect, the antidepressant agent, bupropion, which inhibits both DAT and norepinephrine transporter function (Ascher et al., 1995),

has been shown to be efficacious as a tobacco smoking cessation agent (Hurt et al., 1997; Jorenby et al., 1999; Shiffman et al., 2000). Thus, nornicotine incorporates both inhibition of DAT function and promotion of DA release properties into one molecule, and these pharmacological effects have been associated with the clinical efficacy of the currently available tobacco cessation products, nicotine and bupropion.

Behavioral studies using animal models also provide support for the use of nornicotine as a tobacco use cessation agent. Nornicotine produces nicotine-like discriminative stimulus effects (Rosecrans and Meltzer, 1981; Goldberg, et al., 1989; Bardo et al., 1997; Desai et al., 1999), as well as nicotine-like effects on schedule-controlled operant responding (Risner et al., 1985; 1988). Recent results indicate that nornicotine functions as a positive reinforcer (Bardo et al., 1999); however, under similar experimental conditions, nornicotine is associated with a lower rate of responding in comparison with nicotine (Corrigall and Coen, 1989; Donny et al., 1995; Bardo et al., 1999), suggesting that nornicotine has a lower reinforcing efficacy. Moreover, nornicotine has been shown to decrease self-administration of nicotine in rats (Green et al., 2000). Furthermore, across repeated nornicotine pretreatments, tolerance did not develop to nornicotine-induced decrease in nicotine self-administration. Thus, a simple structural change, i.e., removal of the N-methyl group from the pyrrolidine ring nitrogen of nicotine which affords nornicotine, causes a profound change in its effect on DAT function, which may be beneficial with respect to its pharmacological profile. In

summary, the current preclinical results suggest that nornicotine could be a promising candidate for development as a smoking cessation agent.

X. Future Directions

The current series of experiments demonstrate that nicotine, via a nicotinic receptor-mediated mechanism, increased DA clearance in striatum and MPFC. It is possible that the difference in the response to nicotine between these brain regions is due to the involvement of different nicotinic receptor subtypes. Thus, future studies using various subtype selective antagonists to inhibit the effect of nicotine on DAT function would help determine which subtypes are involved in modulating DAT.

In the current experiments, nicotine was administered systemically. Incubation of synaptosomes from drug naïve animals in vitro with nicotine did not result in an increase in the V_{\max} for [^3H]DA uptake. Because systemic administration of nicotine increased both the V_{\max} for DA uptake in vitro and increased DA clearance in vivo, to determine if the effect of nicotine is occurring in the striatum, nicotine could be locally applied into the striatum and DA clearance determined. Therefore, the effect of nicotine could be localized to the striatum. Conversely, if local application of nicotine did not increase DA clearance, it would suggest the role of circuitry in the effect of nicotine. Another potential way to determine if the effect of nicotine is occurring locally, slices from the brain region could be removed and voltammetry performed in the slices. Use

of slices would allow determination of the localization of nicotinic receptors either on the DA nerve terminals or if other neurotransmitter systems that may alter DAT function.

The increase in the V_{\max} for [^3H]DA uptake was observed in the absence of an increase in trafficking of DAT to the cell surface membrane. The increase in V_{\max} was approximately 25%, which is at the limit of detection for biotinylation experiments. It is possible that increasing the dose of nicotine would lead to a further increase in the V_{\max} . If the V_{\max} further increases, it is possible that there will be a change in DAT trafficking. If no change in DAT trafficking persists, it is likely that the increase in DAT function is due to the involvement of another neurotransmitter system or specific second messenger systems. Use of slices in voltammetric experiments would allow determination of the different mechanisms involved in nicotine modulation of DAT function.

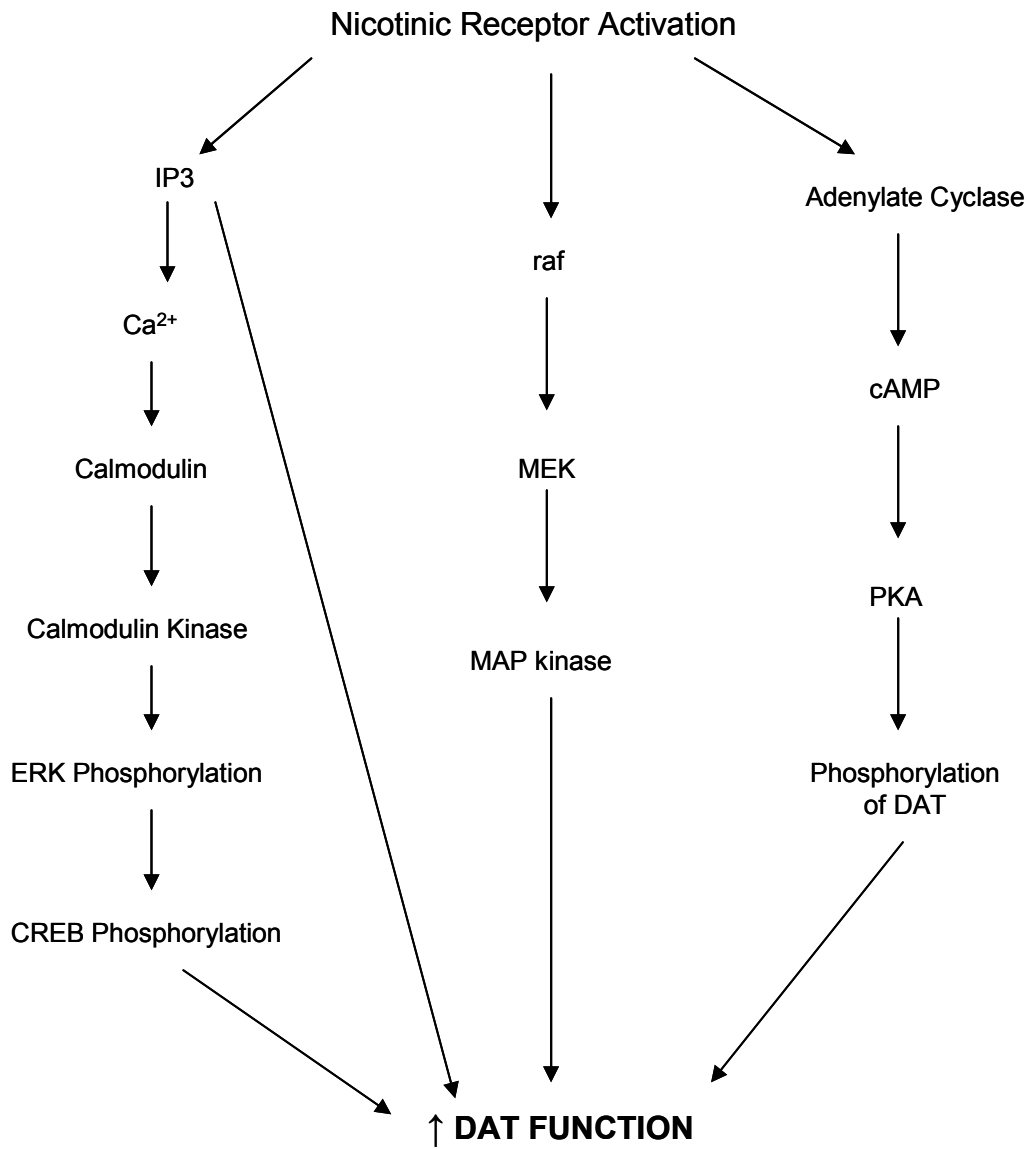
One major difference between the in vivo and in vitro experiments is the use of anesthetic. While the anesthetic used in the current experiments, urethane, does not appear to affect DAT function (Sabeti et al., 2003), it is possible that it is altering other systems that could impact the effect of nicotine on the DA system. To rule out the caveat of using an anesthetic, the effect of nicotine on DAT function could be measured using voltammetry in awake animals.

Finally, the effect of nicotine to increase DAT function by increasing the V_{\max} for DA uptake is important to determine in MPFC. If there is an increase in V_{\max} , then the ability of nicotine to modulate trafficking of DAT would be important to determine. The limiting factor for using MPFC for the in vitro and trafficking experiments is that it requires the use of a large number of animals to obtain enough protein to see any effects.

XI. Final Comments

The results of the current experiments in this dissertation project are the first to examine the effects of nicotine alone on DAT function in striatum and MPFC. Furthermore, this is the first report examining the mechanism by which nicotine does modulate DAT. Nicotine action on the DA system has been well studied with regards to DA release. However the impact of nicotine action on DAT will be an important mechanism that has to be considered not only in how nicotine acts but in the treatment of nicotine addiction.

Figure 24. Schematic representation of second messenger pathways involved in nicotinic receptor modulation of DAT function.



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Manuscripts:

Middleton LS, Cass WA and Dwoskin LP (2004) Nicotinic receptor modulation of dopamine transporter function in striatum and medial prefrontal cortex in rats. *J Pharmacol Exp Ther* 308(1):367-77.

Neugebauer NM, Bryant RI, Cunningham ST, Zhu J, **Middleton LS** and Dwoskin LP (2004) Environmental enrichment mitigates the behavioral and neurochemical effects of prenatal cocaine in adult rats. *Brain Research* 153:213-223.

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Grants and Fellowships:

Petite Grant, Center for Drug and Alcohol Abuse. "Nicotinic receptor modulation of the dopamine transporter." P.I. **LS Middleton**. University of Kentucky, 07/00 – 07/02. \$4,000.00

NIDA T32 DA 07304 "Research Training in Drug Abuse Behavior." Predoctoral traineeship position, Department of Behavioral Sciences, College of Medicine, University of Kentucky, 07/00 – 07/02. \$31,560

National Research Service Award. DA15292 "Nicotinic Receptor Regulation of Dopamine Transporters." P.I. **L.S. Middleton**. 05/02 – 05/04, \$37,000.

Awards

Target of Opportunity Research Award (TORA), Graduate School, University of Kentucky, 08/99 – 05/00.

American Society for Pharmacology and Experimental Therapeutics (ASPET)
Graduate Student Travel Award to attend EB01 in Orlando, FL. 04/01.

American Society for Pharmacology and Experimental Therapeutics (ASPET)
Best Paper Award. “Nicotinic receptor modulation of dopamine transporter function in striatum and medial prefrontal cortex in rats.” EB01 in Orlando, FL. 04/01.

American Society for Pharmacology and Experimental Therapeutics (ASPET)
Graduate Student Travel Award to attend EB02 in New Orleans, LA. 04/02.

Frontiers in Addiction Biology Student Travel Award to attend Frontiers in Addiction Biology in Nashville, TN. 06/04.

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